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 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 thermophilic 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 thermophilic 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.

Thermophilic bacteria were enumerated after heating milk (5 mL) to a temperature that stimulates pasteurization ($62.8 \pm 0.5^\circ\text{C}$ 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 $35 \pm 1^\circ\text{C}$ 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 thermophilic 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, $1\times$ 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), *TaqI* (Ludwig Biotech, Santa Maria, Brazil), *RsaI* (Invitrogen), and *HaeIII* (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-

Table 1. Identification, proportionality, and spoilage potential of thermophilic bacteria isolated from 20 Brazilian milk samples

Identification	GenBank accession no.	Total, no. (%)	Proteolytic and lipolytic, no. (%)	Proteolytic, no. (%)	Lipolytic, no. (%)
<i>Bacillus licheniformis</i>	MG561890	45 (34.1)	16 (35.6)	11 (24.4)	18 (40)
<i>Brachybacterium nesterenkovi</i>	MG561891	19 (14.4)	5 (26.3)	5 (26.3)	9 (47.4)
<i>Enterococcus faecalis</i>	MG561892	12 (9.1)	7 (58.3)	2 (16.7)	3 (25)
<i>Streptococcus agalactiae</i>	MG561893	11 (8.3)	1 (9.1)	9 (81.8)	1 (9.1)
<i>Bacillus</i> spp.	MG561894	6 (4.55)	3 (50)	2 (33.3)	1 (16.7)
<i>Micrococcus aloeverae</i>	MG561895	6 (4.55)	5 (83.3)		1 (16.7)
<i>Streptococcus thermophilus</i>	MG561896	6 (4.55)		6 (100)	
<i>Kocuria varians</i>	MG561897	5 (3.9)	2 (40)	1 (20)	2 (40)
<i>Bacillus circulans</i>	MG561898	4 (3.0)	1 (25)	1 (25)	2 (50)
<i>Paenibacillus cookii</i>	MG561899	4 (3.0)			4 (100)
<i>Kocuria kristinae</i>	MG561900	3 (2.3)	1 (33.3)	1 (33.3)	1 (33.3)
<i>Bacillus clausii</i>	MG561901	2 (1.5)			2 (100)
<i>Bacillus invictae</i>	MG561902	2 (1.5)			2 (100)
<i>Paenibacillus puldeungensis</i>	MG561903	2 (1.5)		1 (50)	1 (50)
<i>Streptococcus gallolyticus</i>	MG561904	2 (1.5)		1 (50)	1 (50)
<i>Kocuria</i> spp.	MG561905	1 (0.75)	1 (100)		
<i>Macrococcus caroselicus</i>	MG561906	1 (0.75)	1 (100)		
<i>Paenibacillus wynnii</i>	MG561907	1 (0.75)		1 (100)	
Total		132 (100)	43 (32.6)	41 (31)	48 (36.4)

file were analyzed by the software Bionumériques v. 1.50 (Applied Mathematics, Kortrijk, Belgium). The Dice similarity coefficient (Dice, 1945) and the unweighted pair group mean averages algorithm (Sneath and Sokal, 1973) were used. To determine the clusters, a minimum of 50% phylogenetic similarity was used.

For DNA sequencing, the Sanger method (ABI 3500 Genetic Analyzer, Applied Biosystems, Foster City, CA) was used in both directions. For this, a representative isolate from each cluster was randomly selected for a new PCR reaction of the 16S rRNA gene and was subjected to purification (PureLink Genomic DNA Purification Kit, Invitrogen) and quantification (Qubit dsDNA HS Assay Kit, Invitrogen).

The quality of the sequences was evaluated by BioEdit v. 7.2.5 (Hall, 1999), and consensual sequences were generated by CAP 3 (Huang and Madan, 1999). Preliminary identification at the genus level was performed using the Blast tool from the National Center for Biotechnology Information (Bethesda, MD). Sequences were individually aligned by Clustal W with representative strains for all the species of each genus available in the Ribosomal Database Project. The neighbor joining method and the Tamura-Nei model using 1,000 bootstraps in Mega v. 7.0 (Kumar et al., 2016) were used to identify the species by genetic similarity. A representative 16S rRNA gene sequence of each identified strain was deposited on GenBank.

RESULTS

Thermophilic bacteria were quantified and isolated from raw milk samples following the exposure of the

milk to pasteurization-like conditions. The thermophilic counts varied between 5 cfu/mL (milk sample 14) and 1.9×10^3 cfu/mL (milk sample 11), with a mean of $3.2 (\pm 4.7) \times 10^2$ cfu/mL for the 20 evaluated milk samples. From the plates used for the counts, 310 colonies were selected and confirmed to represent single isolates by passage on nutritive agar medium, of which 132 (42.6%) had milk-deteriorating potential. Of these, 43 (32.6%) were proteolytic and lipolytic simultaneously, 41 (31%) were exclusively proteolytic, and 48 (36.4%) were only lipolytic (Table 1).

These 132 isolates of potentially thermophilic milk-deteriorating bacteria were grouped by RFLP according to their proteolytic or lipolytic potential in 2 dendrograms. Figure 1 shows the grouping of the proteolytic thermophilic microorganisms, and Figure 2 shows the grouping of the lipolytic microorganisms, representing 41 (I to XLI) and 38 (I to XXXVIII) RFLP types, respectively.

From the representative isolates from the clusters selected for DNA sequencing, identification at the species level by Clustal W and phylogenetic analysis was possible in 94.7% of the 132 thermophilic isolates; of the remaining 7 isolates, 6 were identified as *Bacillus* and 1 was identified as *Kocuria*. The composition of the potential thermophilic microbiota that may contribute to the spoilage of the milk samples is detailed in Table 1.

Regarding the diversity, 8 genera (*Bacillus*, *Brachybacterium*, *Enterococcus*, *Streptococcus*, *Micrococcus*, *Kocuria*, *Paenibacillus*, and *Macrococcus*) were found to be milk-deteriorating thermophilic bacteria from the 20 Brazilian milk samples studied by this work. All were

Figure 1. The RFLP data of the proteolytic thermophilic bacteria isolated from 20 Brazilian milk samples, elaborated using enzymatic digestion of the 16S rRNA gene with the enzymes *Cfo*I, *Taq*I, *Hae*III, and *Rsa*I, the Dice coefficient, and the unweighted pair group mean averages algorithm. The vertical line represents the minimum percentage of similarity (50%) used to determine the 41 RFLP types.

Figure 2. The RFLP data of the lipolytic thermophilic bacteria isolated from 20 Brazilian milk samples, elaborated using enzymatic digestion of the 16S rRNA gene with the enzymes *Cfo*I, *Taq*I, *Hae*III, and *Rsa*I, the Dice coefficient, and the unweighted pair group mean averages algorithm. The vertical line represents the minimum percentage of similarity (50%) used to determine the 38 RFLP types.

Table 2. Distribution of RFLP types and identification of potentially milk proteolytic thermophilic bacteria from 20 Brazilian pasteurized milk samples

RFLP type	Identification	Milk sample (no. of isolates)	RFLP type	Identification	Milk sample (no. of isolates)
I	<i>Bacillus</i> spp.	2 (1)	XXV	<i>B. licheniformis</i>	10 (2)
		9 (1)			16 (1)
		10 (1)			19 (1)
		20 (2)			20 (2)
II	<i>Bacillus licheniformis</i>	20 (1)	XXVI	<i>K. varians</i>	18 (1)
III		10 (1)	XXVII	<i>S. agalactiae</i>	9 (1)
		17 (1)			10 (1)
IV		16 (1)			18 (1)
V	<i>Kocuria kristinae</i>	1 (1)	XXVIII	<i>B. licheniformis</i>	19 (3)
VI	<i>Micrococcus aloeverae</i>	6 (1)	XXIX		16 (1)
VII	<i>K. kristinae</i>	1 (1)	XXX		9 (1)
VIII	<i>M. aloeverae</i>	10 (1)			17 (2)
		12 (1)			18 (2)
IX		5 (1)	XXXI	<i>Brachybacterium nesterenkovi</i>	9 (1)
X	<i>B. licheniformis</i>	16 (1)			10 (1)
XI	<i>Kocuria varians</i>	4 (1)			19 (1)
XII	<i>B. licheniformis</i>	9 (1)	XXXII	<i>K. varians</i>	19 (1)
XIII	<i>M. aloeverae</i>	3 (1)	XXXIII	<i>Paenibacillus puldeungensis</i>	15 (1)
XIV	<i>Kocuria</i> spp.	15 (1)	XXXIV	<i>B. nesterenkovi</i>	1 (1)
XV	<i>B. licheniformis</i>	10 (2)			4 (2)
		11 (1)			6 (1)
		19 (1)			9 (1)
XVI	<i>Paenibacillus wynnii</i>	9 (1)	XXXV		16 (1)
XVII	<i>Enterococcus faecalis</i>	19 (1)			19 (1)
		20 (4)	XXXVI	<i>E. faecalis</i>	2 (1)
XVIII		12 (1)	XXXVII	<i>Streptococcus thermophilus</i>	19 (2)
XIX	<i>Bacillus circulans</i>	4 (1)	XXXVIII	<i>S. agalactiae</i>	2 (1)
XX		7 (1)			19 (1)
XXI	<i>Streptococcus agalactiae</i>	19 (2)	XXXIX	<i>S. thermophilus</i>	1 (4)
XXII		5 (1)	XL	<i>Macrococcus caroselicus</i>	9 (1)
XXIII	<i>E. faecalis</i>	12 (1)	XLI	<i>Streptococcus gallolyticus</i>	8 (1)
XXIV	<i>S. agalactiae</i>	9 (2)			

gram-positive microorganisms with known thermophilic potential.

The species *Bacillus licheniformis*, *Brachybacterium nesterenkovi*, *Enterococcus faecalis*, and *Streptococcus agalactiae* predominated all the potential milk-deteriorating thermophilic bacteria. *Bacillus licheniformis*, *E. faecalis*, *Br. nesterenkovi*, and *Micrococcus aloeverae* represented 76.7% of the bacteria that were simultaneously potential proteolytic and lipolytic thermophilic microorganisms. *Bacillus licheniformis*, *S. agalactiae*, and *Streptococcus thermophilus* were among the predominant bacteria that were exclusively proteolytic. Among the exclusively lipolytic bacteria, *B. licheniformis*, *Br. nesterenkovi*, and *Paenibacillus cookii* predominated.

In relation to each RFLP type, the microbial diversity is detailed in Tables 2 and 3 according to the proteolytic and lipolytic potential, respectively. The same bacterial species from the same milk samples had different RFLP types, unlike the same RFLP types that were observed in the microorganisms from different milk samples.

Milk sample 8 had the lowest diversity among the potentially milk-deteriorating thermophilic bacteria, from

which only 2 strains of the genus *Bacillus* (*B. licheniformis* and *B. circulans*) were isolated. This sample, therefore, contributed only 1.5% of the total microbiota evaluated by this work (132), as well as sample 14, which presented *B. licheniformis* and *S. agalactiae* as potentially milk-deteriorating thermophilic bacteria and thus had greater diversity in relation to sample 8.

The greatest diversity was observed in milk samples 9 and 19, which presented 7 different species of potentially deteriorating thermophilic bacteria (Tables 2 and 3). It is also worth mentioning that the species *B. licheniformis* represented 40% of the isolates in sample 19, from which the highest number of potentially deteriorating thermophilic bacteria were recovered (n = 15).

DISCUSSION

The mean total bacterial count in the present sample was 1.5×10^4 cfu/mL (Ribeiro Júnior et al., 2018). Therefore, the average thermophilic count obtained in this study corresponded to only 2.1% of the total microorganisms present. The thermophilic counts observed in this work (2.5 log cfu/mL) were lower than

in the study by Buehner et al. (2014), which found a mean of $2.76 \log \text{ cfu/mL}$ in 10 samples of refrigerated raw milk in South Dakota in the United States.

Some dairy industries, especially in New Zealand, use a thermophilic count of $1.5 \times 10^2 \text{ cfu/mL}$ in raw milk as the limit in milk quality payment programs. Only 1 milk sample (number 11) in the present study had a higher score than the New Zealand standard ($2 \times 10^3 \text{ cfu/mL}$), reaffirming the high quality of the milk produced in the studied region.

Regarding the microbial diversity, *B. licheniformis* was the most frequent species among the spoilage thermophilic microorganisms and was isolated from 16 different milk samples (Tables 2 and 3), with most isolates (40%) producing lipases, and 35.6% that simultaneously had proteolytic and lipolytic potential (Table 1). Of the 45 isolates of this species, which can be observed in Table 2, 10 RFLP types were classified in the dendrogram of potentially proteolytic thermophilic bacteria (Figure 1) and in 9 of the potentially lipolytic bacteria (Table 3).

Among the potential proteolytic bacteria, it is possible to verify that *B. licheniformis* isolates that make up RFLP types III, XV, XXV, and XXX (Figure 2) comprise strains that are isolated from different milk samples, which may indicate that different milk samples may have *B. licheniformis* contamination from the same source.

In addition, milk sample 16 showed the highest diversity among the *B. licheniformis* strains: 4 different RFLP types (IV, X, XXV, and XXIX) were observed in the dendrogram of the proteolytic microorganisms (Table 2). That is, the same milk sample can also be contaminated by different strains of the same species because several papers conclude that microbial spores can contaminate milk via the soil (Vissers et al., 2007), silage (Hull et al., 1992; Buehner et al., 2014), water (Torp et al., 2001), feces (Hull et al., 1992), teats (Christiansson et al., 1999), and milking equipment (te Giffel et al., 2002).

Bacillus licheniformis is often reported as an endospore-forming agent and is the most frequent bacterial

Table 3. Distribution of RFLP types and identification of potentially milk lipolytic thermophilic bacteria from 20 Brazilian pasteurized milk samples

RFLP type	Identification	Milk sample (no. of isolates)	RFLP type	Identification	Milk sample (no. of isolates)
I	<i>Bacillus licheniformis</i>	9 (1)	XX	<i>B. licheniformis</i>	20 (1)
		17 (3)	XXI	<i>Enterococcus faecalis</i>	1 (1)
		18 (2)			17 (1)
II	<i>Kocuria varians</i>	13 (1)			20 (1)
III		4 (2)	XXII		1 (1)
IV	<i>B. licheniformis</i>	10 (2)			19 (1)
		14 (1)			20 (3)
		15 (1)	XXIII		12 (1)
		19 (1)	XXIV	<i>Bacillus</i> spp.	10 (1)
		20 (2)			19 (1)
V	<i>K. varians</i>	18 (1)	XXV	<i>E. faecalis</i>	12 (1)
VI	<i>Paenibacillus puldeungensis</i>	16 (1)	XXVI	<i>Bacillus circulans</i>	4 (1)
VII	<i>B. licheniformis</i>	18 (1)			8 (1)
		19 (1)	XXVII		17 (1)
VIII	<i>Paenibacillus cookii</i>	11 (4)	XXVIII	<i>B. licheniformis</i>	3 (2)
IX	<i>B. licheniformis</i>	12 (1)			6 (1)
X		10 (2)	XXIX	<i>Kocuria kristinae</i>	1 (1)
		17 (1)	XXX	<i>Streptococcus agalactiae</i>	19 (1)
		20 (2)	XXXI		19 (1)
XI	<i>Bacillus</i> spp.	13 (1)	XXXII	<i>B. licheniformis</i>	16 (1)
		20 (1)			17 (3)
XII	<i>Brachybacterium nesterenkovi</i>	10 (1)			19 (2)
		12 (1)			20 (1)
XIII	<i>Bacillus clausii</i>	1 (1)	XXXIII	<i>Streptococcus gallolyticus</i>	15 (1)
		7 (1)	XXXIV	<i>B. nesterenkovi</i>	4 (5)
XIV	<i>Micrococcus aloeverae</i>	7 (1)			5 (2)
XV	<i>B. licheniformis</i>	15 (1)	XXXV		9 (1)
		16 (1)			10 (1)
XVI	<i>Kocuria</i> spp.	13 (1)			19 (2)
XVII	<i>Bacillus invictae</i>	10 (1)			20 (1)
		19 (1)	XXXVI	<i>M. aloeverae</i>	20 (1)
XVIII	<i>M. aloeverae</i>	3 (1)	XXXVII	<i>Macrococcus caroselicus</i>	2 (1)
XIX		5 (1)	XXXVIII	<i>K. kristinae</i>	10 (1)
		11 (1)			
		12 (1)			

spore producer in milk in several countries (Coorevits et al., 2008; Reginensi et al., 2011; Yuan et al., 2012; Buehner et al., 2014). The bacteria can also multiply at refrigeration temperatures (Doyle et al., 2015; Ribeiro Júnior et al., 2018), allowing them to develop and endure through the milk chain. The fact that the bacteria are sporulated makes their presence even more worrisome because the germination of these spores determines the reduction of shelf life of the pasteurized milk (Huck et al., 2007).

Other microorganisms that are generally described as endospore formers have been described in the thermophilic microbiota in the present work, such as *Bacillus circulans* and *Paenibacillus*. The first description of the isolation of microorganisms of the genus *Paenibacillus* in Brazilian refrigerated raw milk was recently reported by the team from the present study (Ribeiro Júnior et al., 2016a). This genus was isolated from 4 milk samples (9, 11, 15, and 16) that were evaluated in this study (Tables 2 and 3) in 2 different RFLP types for proteolytic (XVI and XXXIII) and lipolytic (VI and VIII) bacteria.

The studies by Huck et al. (2007) and Durak et al. (2006) reported that *Bacillus* and *Paenibacillus* represent the greatest potential for reducing the shelf life of pasteurized milk after 14 d, which reaffirms the importance of keeping the contamination of the milk by these microorganisms under control. In the present work, *Bacillus* and *Paenibacillus* account for 50% (66 isolates) of potential spoilage thermophilic microorganisms in the 20 milk samples evaluated. Only 1 milk sample (5) did not present any *Bacillus* or *Paenibacillus* isolates, meaning that 19 (95%) samples did present these genera. As an alternative for the control of these thermophilic microorganisms, the study by Khanal et al. (2014) reports that the ultrasonication of milk can eliminate the vegetative forms of the thermophilic microorganisms of the genus *Bacillus*.

Brachybacterium nesterenkovi was the second most frequent species that was isolated from the thermophilic spoilage microorganisms in this work (14.4%), with a predominance of strains with lipolytic activity (47.4%; Table 1). No previous reports on its thermophilic capacity and milk spoilage potential were found. This species was identified in 10 (1, 4, 5, 6, 9, 10, 12, 16, 19, 20) of the 20 samples evaluated in the present work. Among the thermophilic bacteria with proteolytic potential (Table 2), 3 RFLP types (XXXI, XXXIV, and XXXV) were observed, and among the potentially lipolytic (Table 3) bacteria, we identified 3 other RFLP types (XII, XXXIV, and XXXV). Thus, although they have a high prevalence in the milk samples, the diversity of *Br.*

nesterenkovi strains is lower in relation to *B. licheniformis*, being observed in possible clonal populations in different milk samples based on RFLP types.

Enterococcus faecalis also predominated among the potentially deteriorating thermophilic bacteria of this sample unit, being isolated in 7 milk samples (1, 7, 12, 17, 18, 19, and 20). Of the total isolates of this species, 42% were identified only in milk sample 20 (Table 2). Possible clonal populations of this species were identified in milk samples 19 and 20 (RFLP type XVII in Figure 1); samples 1, 17, and 20 (XXI RFLP type in Figure 2); and samples 5, 19, and 20 (RFLP type XXII on Figure 2).

A study by McAuley et al. (2012) reported the thermophilic potential of several species of *Enterococcus*, including the species *E. faecalis* that was identified by the present work. Another study by the same authors (McAuley et al., 2015) reported that *Enterococcus* is present in 96% of raw milk samples in Australia, and *E. faecalis* is the most common species (74.3% of 909 isolates).

A study by Delgado et al. (2013) verified the presence of *E. faecium*, *S. thermophilus*, *Kocuria* spp., *Streptococcus* spp., and other lactic acid bacteria in pasteurized milk. Some of the autochthonous lactic acid bacteria of raw milk can withstand pasteurization, which is desirable in many technological processes in the production of dairy products (Ortolani et al., 2010) or undesirable if in fluid milk, causing deterioration. *Micrococcus* and *Streptococcus* species are often isolated from pasteurized milk (Hull et al., 1992), and cases of mastitis in the herd may be a source of milk contamination by these microorganisms (Taponen et al., 2017).

Flint et al. (1999) also reported the thermophilic potential of *S. thermophilus* and its ability to form biofilms. According to the authors, this species may compromise the quality of pasteurized milk. Six (100%) strains of this species were identified in the present study; the strains were isolated from 2 different milk samples (1 and 19), had proteolytic activity, and were identified as 2 distinct RFLP types (XXXVII and XXXIX) and possible clonal populations in each milk sample.

In addition, biofilm-forming thermophilic microorganisms, such as *S. thermophilus*, can adhere to the heat exchanger plates of dairy equipment and compromise the quality of the milk processed therein. The study by Knight et al. (2004) proposes cycles to control biofilms to obtain *S. thermophilus*-free pasteurized milk.

Notably, the present work evaluated the mesophilic thermophilic microbiota. Some microorganisms, including spores, may be thermophilic, deteriorating and multiplying in refrigerated temperatures, and are thus

psychotropic and thermophilic. The study by Ribeiro Júnior et al. (2017) isolated *Bacillus pumilus*, an endospore-forming bacterium (Buehner et al., 2015) with proteolytic potential, from milk after pasteurization and incubation at 7°C for 10 d. The same study also isolated fungal microorganisms in the psychotropic thermophilic spectrum, such as *Cladosporium cladosporioides*, *Geotrichum candidum*, and *Curvularia geniculatus*, all with proteolytic activity, demonstrating the thermal amplitude to which these microorganisms adapt as well as the diversity of this microbiota.

Considering the resistance of thermophilic bacteria to heat treatments and the high percentage of sporulates within them, the best strategy to increase milk quality and its technological potential is to avoid contamination by thermophilic microorganisms during the milking, storage, and transport of raw milk. Therefore, other studies are needed to find the sources of milk contamination by these microorganisms under Brazilian production conditions and to establish strategies to avoid milk contamination.

CONCLUSIONS

Even in raw milk obtained with low total counts of microorganisms, several thermophilic bacteria have proteolytic or lipolytic potential that can compromise the quality of pasteurized milk. These bacteria are mainly *Bacillus* and other endospore-forming species as well as other nonsporulated bacteria, which can be resistant to milk heat treatments. Therefore, the best way to reduce the presence of these microorganisms in pasteurized milk is to avoid their inclusion in raw milk.

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