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# Thermoduric psychrotrophic proteolytic microbiota from refrigerated raw milk

# Microbiota termodúrica psicrotrófica proteolítica do leite cru refrigerado

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## Abstract

Thermoduric microorganisms may withstand high temperatures during the pasteurization of milk. Therefore, the microbiota in pasteurized milk consist of thermoduric microbes and directly influence the shelf-life of the milk. The aim of this study was to identify thermoduric psychrotrophic proteolytic microbiota in refrigerated raw milk. Twenty samples, previously heat-treated, were streaked and incubated at 7°C for 10 days. The strains isolated were streaked on milk agar to assess proteolytic activity and were initially analyzed morphologically by light microscopy and then by molecular techniques to identify the species. In 40% of the samples analyzed was observed only one bacterial growth and others 10 thermoduric psychrotrophic fungi. All isolates were proteolytic. The sequencing of 16S rRNA gene identified the bacterial strain as *Bacillus pumilus* and analysis of the ITS1-5.8S-ITS2 region of fungal isolates revealed the *Cladosporium cladosporioides* (60%), *Curvularia geniculatus* (10%), and the 3 remaining strains were identified as *Geotrichum candidum* (30%). This is a fist description of *B. pumilus* in Brazilian raw milk. Considering the spoilage potential of all isolates and of the fungi present in raw milk and their survival in pasteurized milk, it is extremely important to carry out further studies to your resistant to heat, their impact on the shelf-life of pasteurized milk, ultra-high temperature (UHT) milk and dairy products.

**Key words**: *Bacillus pumilus*. *Cladosporium*. *Curvularia*. *Geotrichum*. Proteolysis.

## Resumo

Os micro-organismos termodúricos são aqueles capazes de resistir ao processo de pasteurização do leite. Assim, a microbiota do leite pasteurizado é constituída por esses micro-organismos que podem influenciar diretamente na vida sua vida útil. O objetivo do presente trabalho foi identificar a microbiota termodurica psicrotrófica proteolítica do leite cru refrigerado. Foram avaliadas 20 amostras de leite cru refrigerado, plaqueadas e incubadas a 7°C por 10 dias. As cepas isolados foram repicadas em ágar leite para verificação da atividade proteolítica e inicialmente avaliadas microscopicamente e posteriormente identificadas por sequenciamento de DNA. Foi observado apenas um crescimento bacteriano e outros 10 isolados fúngicos termodúricos psicrotróficos em 40% das amostras avaliadas. Todos os isolados foram proteolíticos. O sequenciamento parcial do gene 16S rRNA da cepa bacteriana permitiu a identificação de *Bacillus pumilus* e a análise da região ITS1-5.8S-ITS2 dos isolados fúngicos os

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identificou como *Cladosporium cladosporioides* (60%), *Curvularia geniculatus* (10%), e *Geotrichum candidum* (30%). Não foram encontrados estudos anteriores sobre a presença de *B. pumilus* no leite cru brasileiro. Considerando o potencial deteriorante de todos os isolados e presença de fungos termodúricos psicrotróficos no leite cru, é importante a continuidade trabalhos que avaliem sua resistência térmica e seu impacto na vida útil do leite pasteurizado, longa vida e derivados.
**Palavras-chave:** *Bacillus pumilus*. *Cladosporium*. *Curvularia*. *Geotrichum*. Proteólise.

## Introduction

Thermoduric microorganisms may survive short periods of exposure to high temperatures, as in the pasteurization of milk (JAY, 2000). These microorganisms, jointly with contaminants acquired after heat processing, comprise the microbiota of milk, which is directly related to quality of the product (RANIERI et al., 2012).

Some thermoduric microorganisms also have the ability to grow and multiply at milk-cooling temperatures and often produce proteolytic and/or lipolytic enzymes that degrade the product. These microorganisms belong to the psychrotrophic group (JAY, 2000). These spoilage activities influence the shelf-life of dairy products, as well as their sensory properties (CHEN et al., 2003). Non-thermoduric psychrotrophic microorganisms are destroy during pasteurization and ultra-high temperature (UHT) treatment, although they produce heat-resistant enzymes that may spoil raw milk. However, thermoduric psychrotrophic microorganisms survive heat treatment, propagate and produce enzymes, thus accelerating spoilage.

Thermoduric microorganisms comprise not only bacteria, but also fungi able to grow at cooling temperatures and produce proteases. Some of these fungi, however, present desirable characteristics for food industry applications, since they impart typical sensory properties to some products (POTTIER et al., 2008). Little is known about the presence of fungi in raw milk, including their heat resistance, adaptation to refrigeration temperatures, and spoilage potential.

Thereby, the aim of this study was to identify thermoduric protease-producing psychrotrophic microbiota isolated from refrigerated raw milk, produced in one of the most important dairy regions of Brazil, using microbiological and DNA sequence analysis.

## Materials and Methods

Twenty raw milk samples produced in the dairy region of Castro, Paraná State, Brazil, were analyzed between November 2013 and May 2014. Milk samples were collected in a mug from milk cooling tanks in the dairy farms. Samples were transported in sterile 500 mL containers and immediately refrigerated at the Animal Products Inspection Laboratory, State University of Londrina, Paraná. Samples were analyzed within 4 h of collection.

Samples for the analysis of thermoduric microorganisms were treated as described by Frank and Youssef (2004). After homogenizing the sample in its transportation vial, 5 mL was removed and added to a sterile screw-capped test tube for heat treatment (62.8 ± 0.5°C) in a water bath for 30 min (timed from the moment the water reached the desired temperature). The temperature was determined using a control tube containing a thermometer. Immediately after the heat treatment, the tubes were immersed in an ice bath until they reached a temperature of 10°C.

Serial diluted pasteurized milk samples were then streaked on standard plate count agar (Oxoid, Hampshire, UK) and incubated for 10 days at 7°C to obtain colonies of psychrotrophic microorganisms. This culture medium was suggested by the low specificity, allowing the growth of all microorganisms, bacterial or fungal.

The isolates were transferred to petri dishes with milk agar (Acumedia, Baltimore, USA) supplemented with a 9:1 diluted sterile solution of 10% reconstituted skim milk powder to detect proteolytic activity and incubated at 25°C for 5 days for fungal and 35 ± 1ºC for 48 hours to bacteria (FRANK; YOUSSEF, 2004).

The isolates obtained from the milk samples were initially evaluated macro and micromorphologically. The bacterial isolate was submitted to Gram stain and fungal were placed on 3-point inoculated plates containing Czapek Yeast Autolysate Agar (CYA) (PITT, 1979) and incubated at 25°C for 7 days. The macromorphological characteristics of the strains were then assessed. For micromorphological observation of fungal isolates, slides were prepared with 70% ethanol and lactic acid.

For genomic DNA extraction, we followed a standard phenol-chloroform protocol. The partial amplification of the 16S rRNA gene of bacterial isolate was performed using primers 27f 1492r (OSBORNE et al., 2005) and ITS1-5,8S-ITS2 region was performed for fungal using the primers ITS1 and ITS4 as described by White et al. (1990), using a PTC-100 thermocycler (MJ Research, Inc., Waltham, MA, USA).

The amplified fragments were purified using Wizard® SV Gel and PCR Clean-Up System Kits (Promega, Madison, WI, USA) and were sequenced directly in both directions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using an ABI 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The quality of the sequences obtained was analyzed using the electropherogram generated by BioEdit software (Ibis Bio-sciences, Carlsbad, CA, USA). The sequences were subsequently compared with those deposited in GenBank and MycoBank (www.mycobank.org). When necessary, sequences were aligned using the Clustal W application in the *Bioedit software. A representative sequence of each species was selected to deposit in the GenBank of National Center for Biotechnology Information (NCBI).*

## Results and Discussion

Of the 20 milk samples analyzed, 8 (40%) showed growth. From these samples, one bacterial and 10 fungal thermoduric psychrotrophic isolates were obtained. All isolates were proteolytic, as assessed by proteolysis-derived halos around the colonies on the supplemented milk agar plates.

As noted above, 60% of the samples show no growth of thermoduric psychrotrophic microorganisms, suggesting that the refrigerated raw milk from region evaluated already has satisfactory microbiological quality for the production of long-life pasteurized milk. The dairy region of Castro, which includes the cities of Arapoti and Carambeí, from which originated the sample units of this work is characterized by high productivity of the herds, specialized properties and highly technified where raw milk usually has microbiological scores less than 10 CFU/mL (RIBEIRO JÚNIOR et al., 2015).

However, all the isolates show proteolytic activity, which may compromise the shelf life of pasteurized milk of 40% the samples, without considering the post-pasteurization contamination.

While efforts of technical and industries are designed to control bacteria in raw milk, yeast and filamentous fungi may compose 90.9% of thermoduric psychrotrophic microbiota of milk, as observed in this study, and also present deteriorating activity milk, reducing your shelf-life.

In microscopy analysis the bacterial isolate show mor*phology of Gram positive bacillus, being possible the view of medial endospores. Filamentous fungi* isolates to the genera *Cladosporium* (6 isolates) and *Curvularia* (1 isolate). The other 3 psychrotrophic isolates were morphologically unidentifiable due to absence of characteristic conidia and conidiophores, suggesting yeast.

The analysis of DNA sequence of the bacterial isolate revealed *Bacillus pumilus* (Accession in GenBank: KU359036) and the genus identifications based on morphology of fungal strains were confirmed by sequencing. The isolates were identified as *Cladosporium cladosporioides* (60%) (KP769539) and *Curvularia geniculatus* (10%) (KP769537). The 3 morphologically unidentifiable isolates were identified as *Geotrichum candidum* (30%) (KP769540).

A bacterial growth identified as *B. pumilus*, and this species was not previous mentioned in the microbiota of the Brazilian raw refrigerated milk. This microorganism is often described as a member of aerobic spore microbiota of milk in countries like Belgium (COOREVITS et al., 2008), Uruguay (REGINENSI et al., 2011) and United States (BUEHNER et al., 2014). These studies isolated *B. pumilus* from cow milk after heat treatment for 12 minutes at 80°C, which shows superior resistance to heat treatment used in this work. Thus, it is expected that these microorganisms resistant to the pasteurization process in its vegetative and/or sporulated form.

RUZ-PERES et al. (2010) compared the resistance of filamentous fungi and yeast to rapid (72°C for 20 sec) and slow (65°C for 30 min) pasteurization, as well as boiling (98°C) of milk, found that the rapid pasteurization process was the thermal treatment showing the highest resistance index (72.18%) for the yeast and filamentous fungi strains that were tested. Boiling was in second place (15.89%) and slow pasteurization third (0.99%).

In the same study, the authors found that 77.14% of *Geotrichum* strains (35) were resistant to rapid pasteurization, while none was resistant to slow pasteurization, unlike the present work. *Geotrichum* is described as the most prevalent yeast in milk in the few works available about fungi in milk, as 51.5% in Slovenia (TORKAR; VENGUST, 2008) and 76.5 in Spain (JODRAL et al., 1993).

In contrast, some fungi may have desirable technological characteristics, as *Geotrichum candidum* is a yeast on the surface of some varieties of cheese (ELISKASES-LECHNER et al., 1997). *G. candidum* is used as a leavening agent in the dairy industry and colonizes almost all surface cured cheese varieties during the early ripening stage (BERGER et al., 1999), contributing to texture, cohesion and rind thickness (BOUTROU; GUÉGUEN, 2005), as well as introducing the aromatic compounds that are characteristic of some cheese varieties (JOLLIVET et al., 1994). These characteristics of cheese are mainly attributed to extracellular protease produced by *G. candidum* strains (BOUTROU et al., 2002).

However, in raw liquid milk intended for pasteurization and marketing, these microorganisms may affect shelf-life due to their thermoduric, psychrotrophic, and proteolytic properties. Thus, specific studies of the fungal microbiota in the Brazilian milk and your effect on the shelf life of pasteurized milk are required in order to establish strategies to control or eliminate these spoilage microorganisms.

Was observed that milk with high microbiological quality, this sampling unit, has potential for the production of long shelf-life pasteurized milk, since 60% of the samples no showed growth of thermoduric microorganisms. However, in the other 40% of the samples was observed the predominance of fungi with proteolytic activity. Made the identification of the microbiota, will be possible the establishment of new studies to find the source of these contaminants and developing strategies for their control.

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