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FOOD MICROBIOLOGY - RESEARCH PAPER

Identification and evaluation of thermotolerance of yeasts from milk *in natura* exposed to high temperature and slow and fast pasteurization

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Abstract

Milk is considered one of the basic raw materials of animal origin; it must present hygienic quality and physical–chemical properties suitable for processing and human consumption. Thus, the ingestion of milk *in natura* when not properly treated can be characterized as an opportunistic route of transmission of possible microbial pathogens, which can offer risks to public health. The present study aimed the yeast identification, to analyze the thermo-resistance of yeasts isolated from fresh milk, and to trace the susceptibility profile of the isolates to antifungal agents. For this, 23 samples of fresh milk type B, collected by manual or mechanical milking, were stored in collective refrigeration tanks of farms located in the Metropolitan Region of Natal and nearby, State of Rio Grande do Norte (RN), Brazil. Twenty samples of fresh milk commercially traded in the city of Ceará-Mirim RN were also analyzed. The yeasts were quantified by count of colony-forming units (CFU). All isolated species were treated by slow pasteurization (62–64 °C for 30 min) and fast (72–75 °C for 20 s), as well as by boiling (100 °C). Fifty yeast strains were obtained, and the species were identified as *Candida tropicalis* (28%), *Candida parapsilosis* (14%), *Candida albicans* (12%), *Candida glabrata* (10%), *Candida krusei* (10%), *Kluyveromyces marxianus* (10%), *Candida guilliermondii* (8%), *Candida rugosa* (2%), *Candida orthopsilosis* (2%), *Pichia manshurica* (2%), and *Kodamaea ohmeri* (2%). Five isolates showed resistance to the antifungal agents tested. Among all the isolates submitted to heat treatment, 80% were resistant to fast pasteurization and 60% to boiling, but none of them resisted the slow pasteurization. The milk collected through mechanical milking and stored in collective cooling tanks, presented higher rates of yeast contamination, compared to milk samples collected by manual milking and kept under the same storage conditions.

Keywords Milk · Yeasts · *Candida* · Pasteurization · Boiling

Introduction

Cow milk is an important food that can often be contaminated by yeast, especially those commercialized *in natura*, in cases of mammary or systemic gland infection and even by direct fecal contamination. Thus, bovine milk must be constantly monitored in order to have integrity and quality for human consumption, which is preserved either in its natural form or its derivatives [1, 2].

Currently, the Brazilian population consumes fresh milk and this food may present high counts of microorganisms, especially bacteria, which can cause enterocolitis and are associated with poor hygiene practices of the milkier. Therefore, it is worth mentioning that the presence of possible pathogens is also related to the microbiota of the animal, such bacteria's as the presence of *Staphylococcus aureus*, *Escherichia coli* and some yeasts like *Candida* species, which can pass from the moment of milking to the final consumer, resulting in a lower sanitary quality of milk *in natura* [3, 4].

Hygienic-sanitary measures when not properly or satisfactorily conducted can facilitate the contamination of milk by undesirable microorganisms, which may cause physical and chemical changes in the product and its

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derivatives, limiting its durability, as well as causing economic and public health problems [5].

As a result of the development of yeast in processed foods, especially milk, unpleasant odors, discoloration, texture changes, and gas production can occur which can cause packaging to bulge on refrigerated shelves during their commercial exposure [6].

These microorganisms are important in dairy products in the deterioration of some compounds and the beneficial effects on fermentation of others. The presence of yeasts has been widely described as *Candida* spp., *Kluyveromyces marxianus*, and *Pichia* spp. in dairy products such as milk, cream, butter, as well as yogurt, cheese, and others fermented dairy products [7].

Thus, the heat treatment developed by Louis Pasteur in 1864 is still used to eliminate unsporulated pathogenic microorganisms, as well as to significantly reduce the deteriorating microbiota, causing minimal changes in its chemical composition. Therefore, heating the product to a certain temperature over a period of time, followed by a sudden thermal shock, eliminates the thermosensitive microorganisms, thus increasing the shelf life of the milk, without significantly altering its nutritional and sensory composition [8, 9].

Currently, Brazilian law requires several parameters to evaluate milk quality and udder health, one of them being the Somatic Cell Count (SCC) [2, 10]. In addition, the European Union, China, New Zealand, Australia, Switzerland, and Canada, the legal limit for bulk milk SCC (BMSCC) is $3–4 \times 10^5$ cells/mL; in South Africa and Brazil, 5×10^5 cells/mL; and in the USA, 7.5×10^5cells/mL [11]. However, Brazilian legislation still allows up to 480,000 somatic cells/mL in type A fresh refrigerated milk (when the milk is pasteurized and kept at ≤ 4 °C, preventing microbial multiplication for 24 h) and up to 600,000 somatic cells/mL in fresh refrigerated milk kept in tanks for up to 3 months (type B) [2]. In this way, type A milk is produced under stricter controls, pasteurized and packaged on the farm, and therefore contains fewer microbes; type B milk is sent to the factory where it is pasteurized and packaged; type C milk is also industrially pasteurized and packaged milk, but it contains more microorganisms. In addition, type C milk has a fat content of 3%, while types A and B have a higher fat content [2].

The present study aimed to evaluate the occurrence of yeasts in samples of *in natura* milk and its resistance to temperature, used in the processes of pasteurization and boiling of milk. For this, the isolated yeasts from the collections by manual and mechanical milking were counted, identified phenotypically and genotypically. In addition, the efficiencies of the methods of eliminating microorganisms in food were evaluated, through the processes of boiling, fast and slow pasteurization in order to eliminate potentially pathogenic yeasts.

Materials and methods

Sample yeast collection

A total of 43 milk samples were collected after the final product process; 23 of them from collective refrigeration tanks and dairy properties located in and around the metropolitan region of Natal, Rio Grande do Norte state (RN), Brazil, and the remaining 20 samples from milk *in natura.* Cow milk was required from commercialization in different markets in the city of Ceará-Mirim (RN). The required samples from refrigeration tanks were transported in a cool box containing ice to the Laboratory of Antimicrobial and Cytotoxicity Tests at Federal University of Rio Grande do Norte.

Culture media and yeast cultivation

An aliquot of the biological material was subjected to serial dilutions from 10^{-1} to 10^{-3} in a sterile distilled water (ICMSF 1974). Then, 100 µL of diluted sample was spread on potato dextrose agar medium (Difco Laboratories, Detroit, MI, USA) acidified with 10% tartaric acid (Sigma-Aldrich, Saint Louis, MO, USA) (pH = 4.0) and incubated for 48 h at 37 °C [12]. Malt extract agar should be more suitable than PDA for the cultivation of milk yeast, so there was a limitation for better yeast recovery due to the presence of some differences in pH and nutrients in the medium. Colony-forming units (CFUs) were counted, and subcultures of each sample were identified. Yeast colonies were identified by slide culture on Corn Meal Agar (Himedia Laboratories, Mumbai, India) [13], CHROMagar Candida chromogen medium (BD™, Paris, France) to identify *Candida* suggestive species [14] and confirmation with the DNA sequencing technique.

Antifungal susceptibility

The antifungal susceptibility was obtained with test Fungifast® (ELITech Microbiology Reagents). The kit allows the identification of the main yeasts pathogenic to human, as well as performing sensitivity tests for several antifungal drugs used in the treatment of superficial, dermatomycoses, or systemic mycoses. The antifungal drugs tested were amphotericin B 0.5 µg/mL, amphotericin B 2 µg/mL, 5-flucytosine 4 µg/mL, 5-flucytosine 16 µg/mL, itraconazole 0.125 µg/mL, itraconazole 0.5 µg/mL, fluconazole 8 µg/mL, fluconazole 32 µg/mL, and voriconazole 1 µg/mL.

DNA total extraction and PCR amplification

Genomic DNA was extracted according to a method modified by Looke and collaborators [15] and PCR of the internal transcribed spacer (ITS) region as has been described previously by Godinho and collaborators [16]

using the universal primers ITS1 and ITS4 [17] to identify the microbiota.

DNA sequencing

The PCR product was purified using Kit ExoSAP-IT PCR Clean-up (GE, Healthcare, Sunnyvale, CA) according to manufacturer instructions. The bands purified were sequenced by Sanger method, in a Sequenciator DNA Analayser (Applied Biosystems, Carlsbad, CA). The obtained sequences were analysed with SeqManP with Lasergene software (DNAS-TAR Inc., Madison, WI), and a consensus sequence was obtained using Bioedit ver. 7.0.5.3 software (Carlsbad, ON, Canada). Representative consensus sequences of fungal taxa were deposited into the GenBank database by Nucleotide Basic Local Alignment Search Tool (BLASTN) similarity search algorithm available from the National Center for Biotechnology Information (NCBI).

Nucleotide sequence accession numbers

The ITS sequences isolated from yeasts in this study have been deposited in the GenBank data base under accessio nnumbers MN450866, MN450867, MN4508668, MN450869, MN450870, MN450871, MN450872, MN450873, MN450874, MN450875, MN450876, MN450877, MN450878, MN450879, MN450880, MN450881, MN450882, MN450883, MN450884, andMN450885, as listed in Table 1.

Thermal susceptibility of the yeasts

This test was performed according to the methodology described by Melville et al. [18] with adaptations. From each of the strains of yeast, cell concentrations in sterile saline solution (0.85%) were adjusted in the McFarland 3 scale. The samples were subjected to different temperature/time ratios. The procedures were performed in a water bath, varying the temperature in the following parameters: 62–65 °C for 30 min (slow pasteurization) and 72–75 °C for 20 s (rapid pasteurization). For the boiling process, the five glass tubes were subjected to heat until they reached boiling temperature (98 °C), remaining for the set time of 2 s, and then immediately placed in an ice-water bath. After the temperatures tests, the samples were cultured in Sabouraud-dextrose agar (Difco Laboratories, Detroit, MI, USA) with chloramphenicol (100 µg/mL) using the spread plate technique. The plates were then incubated at 25 °C for 48 h in order to evaluate the count of colony-forming units.

Table 1 Molecular identification of yeast species based on rDNA sequencing and matching with the NCBI GenBank database

Proposed taxa	Top BLAST search results [GenBank accession number]	Query cover (%)	Identity (%)	N° of bp analysed	GenBank accession number
Pichia manshurica	MH393500.1	96%	98%	662	MN450866
Kluyveromyces marxianus	CP023460.1	99%	99%	1002	MN450867
Candida orthopsilosis	LC389313.1	70%	98%	1015	MN450868
Candida parapsilosis	KU961982.1	96%	99%	844	MN450869
Candida parapsilosis	LC389138.1	81%	92%	503	MN450870
Kluyveromyces marxianus	KF646164.1	96%	98%	772	MN450871
Candida parapsilosis	KU200443.1	96%	99%	685	MN450872
Candida parapsilosis	KY178309.1	95%	99%	685	MN450873
Kluyveromyces marxianus	KY103793.1	98%	99%	673	MN450874
Candida parapsilosis	LC389750.1	98%	98%	875	MN450875
Kluyveromyces marxianus	CP015058.1	97%	98%	674	MN450876
Candida tropicalis	MK267747.1	92%	99%	871	MN450877
Kluyveromyces marxianus	KY103808.1	98%	98%	8002	MN450878
Candida tropicalis	MG599234.1	99%	97%	517	MN450879
Candida tropicalis	KX664582.1	94%	98%	1072	MN450880
Candida parapsilosis	LC390144.1	96%	99%	775	MN450881
Kodamaea ohmeri	LC413234.1	81%	98%	333	MN450882
Candida parapsilosis	KX652405.1	98%	99%	892	MN450883
Candida tropicalis	KX664575.1	74%	96%	844	MN450884
Candida rugosa	KJ706251.1	98%	92%	632	MN450885

Statistical analysis

Statistical analysis was performed using Excel software and statistical software R[1]. Initially, a descriptive analysis of the variables was done separately and together with the response variable. Fisher's exact test was analyzed to identify possible associations between the explanatory and response variable. The significance level used in the test decision rule was 5%.

Results

CFU count and yeast characterization

Counting colony-forming units of milk samples revealed an overall median of 2.75×10^3 CFU/mL (ranging from 0.1 to $> 6.0 \times 10^4$ CFU/mL). When analyzed separately, it was found that the milk samples collected on the farms and kept in collective refrigeration tanks, had a median of 3.7×10^3 CFU/mL (ranging from $0.5 > 6.0 \times 10^4$/mL), while those collected in the Ceará-Mirim trade showed a median of 2.0×10^3 CFU/mL (ranging from $4 > 6.0 \times 10^4$ CFU/mL). The results of genotypic identification and sequencing of the twenty strains with the highest CFU counts are shown in Table 1.

The overall percentage of positive milk samples was 74.4%, with 46.5% in milk samples collected from farms and 27.9% in milk samples marketed in Ceará-Mirim (RN). From this total of 32 positive milk samples, 50 yeast strains of the genus *Candida* (43, corresponding to 86% of the strains) were identified, and species *Kluyveromyces marxianus*, *Kodamaea ohmeri*, and *Pichia manshurica*.

Thus, of the 50 isolated yeast strains, 86% correspond to *Candida* species, showing the prevalence of this genus. Furthermore, 50 strains of yeast correspond to the following species: *Candida tropicalis* (28%), *Candida parapsilosis* (14/%), *Candida albicans* (12%), *Candida glabrata* (10%), *Candida krusei* (10%), *Kluyveromyces marxianus* (10%), *Candida guilliermondii* (8%), *Candida rugosa* (2%), *Candida parapsilosis* (2%), *Candida orthopsilosis* (2%), *Pichia manshurica* (2%) *Kodamaea ohmeri* (2%) from 43 milk *in natura* samples.

Antifungal susceptibility

Among of the twenty strains with the highest CFU counts, five presented the resistance patterns determined in the Clinical & Laboratory Standards Institute (CLSI) guide to the antifungal used in the medical clinic. Thus, *C. parapsilosis*: 1 strain showed intermediate resistance (IR) to flucytosine (minimum concentration inhibitory—MIC 8–16 mg/mL) and 1 strain showed intermediate resistant (IR) to Amphotericin B (MIC 2 mg/mL); *C. rugosa*: 1 strain, dose-dependent sensitive (SDD) to fluconazole (MIC 16/32 mg/mL); *C. tropicalis*: 1 strain, intermediate resistant (IR) to amphotericin B (MIC 2 mg/mL); *Kodamaea ohmeri*: 1 strain, dose-dependent sensitive (SDD) to fluconazole (MIC 16/32 mg/mL).

Thermal resistance evaluation of isolates

The evaluation of the susceptibility to temperature and time variation of the 50 strains of yeast isolated, in both pasteurization and boiling processes, revealed that the slow pasteurization at 62–64 °C for 30 min was the most efficient.

On the other hand, rapid pasteurization at 72–75 °C for 20 s showed growth in 80% of isolates. In the milk samples submitted to boiling, there was a 60% growth of the isolates. The evaluated yeasts presented different resistance percentages against these procedures as can be observed in Table 2.

Discussion

Yeasts are considered saprobic and are commonly isolated from milk storage tanks, of which are often unhygienic, which provides for fungal reproduction and identification. In addition, research on dairy yeasts is important because they can cause economic damage by losing the primordial characteristics of dairy products, as well as causing opportunistic mycoses from eating contaminated foods [19]. Thus, it suggests that low sanitary quality or unhealthy conditions of raw milk are indicators for consumer health

Table 2 Frequency of resistance of 50 strains of isolated milk yeasts to slow, rapid and boiling pasteurization processes in percentage

Isolated yeasts	Number of strains tested	Slow pasteurization (%)	Fast pasteurization (%)	Boiling (%)
C. tropicalis	14	0	64.3	43
C. parapsilosis	7	0	100	87
C. albicans	6	0	67	33
C. glabrata	5	0	100	60
C. krusei	5	0	100	40
K. marxianus	5	0	80	80
C. guilliermondii	4	0	75	100
C. rugosa	1	0	0	0
C. orthopsilosis	1	0	100	100
P. manshurica	1	0	100	100
K. ohmeri	1	0	100	100
Total	50	0	80	60

= number of microorganisms identified after each process (pasteurization and boiling)

risks, especially when considering the contamination of coliforms and opportunistic yeasts, which can subsequently present the consumer with an infection of opportunistic character. Yeasts are not dependent beings in the microbiota of raw milk and can be introduced into the milk from the environment, milking equipment during and after milking and from the udder itself [20].

In other studies, conducted in São Paulo state in Brazil, was found lower percentages of positivity regarding the research of *Candida* yeast. *C. albicans* yeast was detected in 8.9% of 260 mastitis cow milk samples [21]. A study from Rio Grande do Sul state, in Brazil, did not detect the presence of *C. albicans* in the milk of animals with clinical and subclinical mastitis, although *Candida* species represent 37.9% of fungal isolates [1].

Mbuk and collaborators [22] investigated the diversity of yeast associated with bovine mastitis using molecular techniques. A total of 300 milk samples were collected from 26 peri-urban farms in Kaduna State, Nigeria, for microbiological analysis. After culture, 37 strains were isolated, and 11 strains of which were genetically identified as *C. albicans* (3 isolates), *Saccharomyces cerevisiae* (1 isolate), and *Pichia kudriavzevii* (7 isolates).

In the present study, five of them showed resistance patterns to only one type of each antifungal. The resistant species *C. parapsilosis* showed the highest resistance profile for flucytosine and amphotericin B; *C. tropicalis* for amphotericin B; and *C. rugosa* and *Kodamaea ohmeri* for fluconazole. Among the other antifungals tested, itraconazole and voriconazole showed no resistance pattern against the strains tested. In the study by Mbuk and collaborators [22], described previously, it was observed the resistance of 22% of fungal isolates to amphotericin B.

The resistance profile showed in *C. parapsilosis* is already known, being this yeast considered the second or third most isolated yeast of blood cultures in the world and it is also quite implicated in fungal infections in Brazil. This is due to its ability to form biofilms in catheters and others medical devices, a factor that contributes to the dissemination via the blood [23].

In a study developed in Yinchuan, China, Du and collaborators [24] reported 60 yeast isolates of 482 milk samples from clinical mastitis cows. Six strains were identified of *C. parapsilosis* showing resistance to flucytosine. Furthermore, two isolates showed resistance to amphotericin B but all strains were susceptible to fluconazole, ketoconazole, and nystatin. So, no strain of *C. albicans* was phenotypically and genotypically identified, corroborating the data from our study.

A divergent result was noted in Minufiya Province, in Egypt, showing that of the 150 milk samples collected from mastitis cows, 29.3% corresponded to *C. albicans* isolates, which can be explained by the use of the species-specific PCR primer, the 26S rRNA gene [25].

According Menezes and collaborators [26], the most important microorganisms are those that contaminate milk during and after milking. This statement was reinforced by the analyzes performed by Santana and collaborators [27] who studied four properties of the region of Londrina, state of Paraná, in Brazil, analyzing various points of the milk production process. Thus, they verified that the main points of contamination were the wastewater from inadequately sanitized brass, expansion tanks, and teats. This fact can corroborate with the need to adopt good hygiene and cleaning practices in milking, which will also contribute to reducing animal breast infections and obtaining a higher quality of microbiological and physicochemical milk.

It is noteworthy that, in this present study, it was found that in the samples obtained from the 20 refrigeration tanks, the global rates of yeast colonies were 3.7×10^3 CFU/mL, being 3.3×10^3 and 8.05×10^3 CFU/mL, for manual and mechanical milking respectively. This higher rate of contamination of milk obtained by mechanical collection may be due to failures in the cleaning process of the equipment used in milking. Thus, there was a higher CFU count of samples collected by mechanical milking and transferred by each producer to the collective refrigeration tanks. This is possibly due to the lack of proper cleaning of the milking equipment.

Another Brazilian study conducted in the state of Goiás, by Souza and collaborators [28], showed that in which samples were collected directly from the local cooperative's milk receiving tank, as well as industrialized milk samples, it was observed the growth of fungal microbiota only in the samples collected from the fresh tank.

In studies with bacterial isolates from raw milk samples in refrigeration tanks, Hahne and collaborators [29] reported a count of 100,000 CFU/mL of bacteria (such as *Acinetobacter* spp., *Pseudomonas* spp., *Streptococcus* spp., and *Escherichia coli*) when evaluating the microbial diversity of 48 samples collected from dairy farms in North Rhine-Westphalia, Westphalia, and Lower Saxony (Germany). The authors described that cold may not always influence the selective pressure of bacteria. Correlated with the present study, fungal growth at low temperatures may depend on species, storage hygiene conditions, and natural competition between strains.

The results in this study showed that the temperature is one of the determining factors to guarantee the growth of the yeasts, as well as the others found, especially in the three different thermal processes: fast, slow pasteurization, and boiling. It was found that the rapid pasteurization process presented the lowest efficiency, followed by boiling. Slow pasteurization was highly efficient as there was no yeast growth after treatment.

In studies of the microbiological profile evaluation of fermented milk, 52 strains were isolated from fermented goat milk from Yaghnob Valley, in Tajikistan (Central Asian), genetically identified using regions ITS1 and ITS4, 29 isolates of *K. marxianus*, *S. cerevisiae* (10), *Pichia fermentans* (12), and *Kazachstania unispora* (1).

The authors reported that *K. marxianus* strains demonstrated two different genotype profiles, in which they differ in variable phenotypic characteristics, such as tolerance to high temperatures, low pH, and presence of acid [30]. These data may explain the thermal resistance seen in *K. marxianus* in the present study, which can also belong to one of the genotypes found in the same previous study.

Ruz-Peres and collaborators [31] conducted a study that aimed to evaluate the resistance to pasteurization and boiling of filamentous fungi and yeasts isolated from fresh cow's milk. They found 27 strains of filamentous fungi and 275 yeasts among them: (50) *C. krusei*, (22) *C. parapsilosis*, (23) *Candida kefyr*, (03) *C. albicans*, (40) *C. guilliermondii*, (14) *Candida lusitaniae*, and (36) *C. tropicalis*. The strains studied were originally isolated from fresh milk from 50 refrigeration tanks and 10 dairy farms, as well as from 10 carriers and distributors that stored and marketed directly to the consumer the milk obtained from the producing properties. These authors found that rapid pasteurization was the procedure with the highest resistance index (72.18%) by yeast and filamentous fungi, followed by boiling (15.89%) and slow pasteurization (0.99%). These results corroborate those found in the present study, demonstrating that rapid pasteurization is not very efficient for eliminating fungi from food for human consumption.

On the other hand, Montanari and collaborators [32] showed that microorganisms exposed to pasteurization and stabilization processes of beverages at high temperatures and prolonged time can exert the survival of strains with high thermal resistance profile. Thus, favoring the selection of strains resistant to stress factors associated with selective pressure in the industrial environment.

An alarming result found in the present study is the heat resistance of rapid pasteurization methodologies and the low efficiency of boiling, the latter being the most used procedure in the practice of most of the Brazilian population as a method of reducing milk contamination for human consumption.

In addition, an important evidence of this study concerns the temperatures of refrigeration tanks, in which milk is stored before being transported to the dairy where it is processed. This temperature should be maintained at a maximum of 4 °C, which is possibly not occurring as higher CFU/mL medians were found in milk samples collected from collective refrigeration tanks, which had a capacity of 500–3000 L. Another possibility (or both situations together) is the lack of proper hygiene during milking processes [2].

Thus, the counting of colony-forming units and phenotypic and genotypic characterizations of yeasts prove to be an indispensable set of methods for monitoring and decreasing the cellular viability of contaminants in food, particularly the milk, thus increasing the validity and availability of food for human consumption.

Conclusion

This study showed the presence of yeasts in fresh milk samples analyzed and in tanks with potential capacity to cause opportunistic mycoses. The identified strains showed a pattern of resistance to antifungals (amphotericin B, fluconazole, and flucytosine). The count of colony-forming units of milk samples obtained by mechanical milking and stored in collective refrigeration tanks was higher than the other types of milk analyzed. Finally, slow pasteurization is more efficient in reducing the yeast microbiota in milk, while fast pasteurization and boiling proved to be inefficient to eliminate these agents present in milk intended for human consumption.

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Author contributions JSC and ACVJ executed, analyzed, and interpreted the data for the manuscript. DB, DLS, and UGPL executed and analyzed the data for the antifungal tests and fungi identification. JVF, MARS, and VSA coordinated all the research and corrections and suggestions in the preparation of the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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