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High frequency of potentially pathogenic yeast species in goat's raw milk and creamed cheese in Southern Brazil

Alta frequência de leveduras potencialmente patogênicas no leite de cabra in natura e no queijo de cabra cremoso no Sul do Brasil

Andréia Spanemberg¹, Jesus Pais Ramos², Orílio Leoncini³, Sydney Hartz Alves⁴ & Patrícia Valente⁵

ABSTRACT

There are few reports concerning isolation, counting and identification of yeasts in goat's raw milk and derivatives. The objective of this study was to evaluate the diversity of yeasts found in raw goat milk and goat creamed cheese collected in a Metropolitan area in Porto Alegre - Brazil. A simplified HMA (Heteroduplex Mobility Assay) of the 26S rDNA D1/D2 region was developed in order to rapidly confirm the identification of the isolates belonging to potentially pathogenic species. Yeasts were isolated from 59% of the samples. Fifty six strains were isolated and identified in the genera *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Pichia*, *Rhodotorula*, *Sporidiobolus*, *Trichosporon*, *Yarrowia* and *Zygoascus*. The average yeast count in raw milk was superior to 2 log UFC.mL⁻¹, while cheese count was superior to 3 log UFC.g⁻¹. Lipolytic activity was present in almost 92% of the isolates, while only 14% had proteolytic activity. Twelve potentially pathogenic ascomycetic isolates were identified by the conventional yeast identification methodology, and correspond to the species *Candida parapsilosis*, *Candida tropicalis* and *Pichia guilliermondii*. All of them had their identities confirmed by the simplified HMA assay. None of the isolates belonging to potentially pathogenic species were resistant to the antifungal agents tested. More studies are necessary to evaluate the real significance of the isolation of these clinically relevant yeasts.

Keywords: yeast identification, goat milk, goat cheese, HMA, *Candida* spp.

RESUMO

Existem poucos relatos sobre isolamento, contagem e identificação de leveduras no leite de cabra *in natura* e derivados. O objetivo desse estudo foi avaliar a diversidade de leveduras encontradas no leite de cabra cru e no queijo cremoso de cabra coletados na região metropolitana de Porto Alegre, Brasil. Foi desenvolvida a técnica de HMA (Heteroduplex Mobility Assay) simplificada da região 26S rDNA D1/D2 para confirmar rapidamente a identificação de isolados pertencentes às espécies potencialmente patogênicas. Leveduras foram isoladas em 59% das amostras. Cinquenta e seis leveduras foram isoladas e identificadas nos gêneros *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Pichia*, *Rhodotorula*, *Sporidiobolus*, *Trichosporon*, *Yarrowia* e *Zygoascus*. A contagem média de leveduras no leite de cabra foi superior a 2 log UFC.mL⁻¹, enquanto que a contagem no queijo foi superior a 3 log UFC.g⁻¹. A atividade lipolítica esteve presente em 92% dos isolados, enquanto que apenas 14% tiveram atividade proteolítica. Doze isolados ascomicéticos potencialmente patogênicos foram identificados pela metodologia convencional de identificação de leveduras e correspondem às espécies *Candida parapsilosis*, *Candida tropicalis* e *Pichia guilliermondii*. Todos tiveram sua identificação confirmada pela técnica de HMA simplificada. Nenhum dos isolados potencialmente patogênicos foram resistentes aos antifúngicos testados. Mais estudos são necessários para avaliar o real significado do isolamento dessas leveduras clinicamente relevantes.

Descritores: identificação de leveduras, leite de cabra, queijo de cabra, HMA, *Candida* spp.

INTRODUCTION

There are few reports concerning isolation, counting and identification of yeasts in raw goat milk and goat cheese. Yeasts represent an important component of the microflora of lactic products, being usually detected in large counts in milk and derivatives due to their richness in proteins, lipids, sugars and organic acids. Besides this, they are capable of growing in substrates with high salt concentrations and low pH [24,28]. Thus they can cause biochemical alterations in food and public health concern [16].

Control of lactic products, in a general way, involves hygienic and sanitary cares that begin in milk production, through adequate milking practices. This fact is relevant because yeasts can be related to cases of mycotic mastitis in goats and cows [10,20], being responsible for economic losses due to the reduction of milk production and augmentation of costs of the production [2,10]. The species of the genus *Candida* are the yeasts more commonly isolated from milk [36]. Some studies report the occurrence and growth of yeasts in several types of cheese and their subproducts [17,35], besides being related to the deterioration of yogurts, concentrated milk and pasteurized milk [13,15].

The objective of this study was to evaluate the diversity of yeasts found in raw goat milk and goat cheese in a Metropolitan area in South Brazil. The HMA technique was used for confirmation of the potentially pathogenic species previously identified by the conventional technique.

MATERIALS AND METHODS

Samples of milk and lactic products

The study was performed with a total of 25 samples, 15 of raw goat milk and 10 of goat creamed cheese from four producers in the Metropolitan region of Porto Alegre during a period of six months. Five milliliters of raw milk from mixtures were aseptically collected in sterile flasks. Samples of cheese were directly collected in the commercial wrappings provided by the producers. All samples were kept under refrigeration and immediately analyzed in the laboratory.

Counting and isolation of yeasts

Serial decimal dilutions were processed from 1mL of goat milk diluted in 9mL of sterile distilled

water, or 10g of cheese samples diluted in 90mL of sterile water. Aliquots of 0.1mL were collected and inoculated in triplicate on modified YM Petri dishes (1% glucose, 0.3% malt extract, 0.5% peptone, 400mg/L chloramphenicol, 2% agar, pH 4.5). After incubation for 3-5 days at 22-25°C, the different morphological colonial types were counted, and representatives of each morphotype were isolated and purified in YEPG medium (1% yeast extract, 2% glucose, 1% peptone, 2% agar). Pure cultures of each strain were maintained on YEPG agar slants covered with sterile mineral oil, and kept in the refrigerator.

Yeast conventional identification

The isolates were identified by means of phenotypic (morphological and biochemical) criteria [4] and by means of the computer program YEASTCOMPARE (C. Ciriello and M.A.Lachance, Copyright© 1999-2001).

Molecular identification

Strains identified by the conventional yeast identification methodology as belonging to potentially pathogenic *Candida* species had their identities confirmed by the heteroduplex mobility assay using the D1/D2 rDNA region [26]. DNA extraction, PCR amplification of the D1/D2 region and HMA assays were performed as described by Ramos *et al.* (2006) with modifications, aiming the simplification of the method. For heteroduplex formation, 100ng of each PCR-amplified D1/D2 region were combined, followed by addition of 10X annealing buffer (1M NaCl; 100mM tris, pH 7.8; 20mM EDTA). DNA mixtures were heated at 94°C for 3 minutes, and rapidly cooled in ice. Homoduplexes and heteroduplexes were separated by electrophoresis on a 5% polyacrylamide gel at constant 250V for 2h30min. The bands on the polyacrylamide gels were stained with ethidium bromide, visualized under U.V.-light and scanned. The type strain of each species was used as the positive control for the HMA assay (*Candida parapsilosis* NRRL Y-12969, *Candida tropicalis* NRRL Y-12968 and *Pichia guilliermondii* NRRL Y-2075), meaning that if the identification was right, there would be no heteroduplex formation when the PCR products of the milk/cheese strain was mixed with it. The negative controls were chosen based on the shortest genetic distance among the strains. For presumptive *C. parapsilosis* strains, the negative control was the type strain of *C. tropicalis*, meaning

that heteroduplex should be formed when their PCR products were mixed. The negative control for presumptive *C. tropicalis* and *Pichia guilliermondii* (anamorph *C. guilliermondii*) was the type strain of *C. parapsilosis*.

Antifungal susceptibility

Standard antifungal powders of fluconazole, itraconazole and amphotericin B were obtained from their respective manufacturers. Stock solutions were prepared in water (fluconazole) and dimethyl sulphoxide (amphotericin B and itraconazole). Serial twofold dilutions were prepared exactly as outlined in NCCLS document M27-A2 [25]. Final dilutions were made in RPMI 1640 medium buffered to pH 7.0 with 0.165 mmol l⁻¹ morpholinopropanesulphonic acid (MOPS) buffer. The final concentration of the solvent did not exceed 1% in any well. Aliquots (100 µL) of each antifungal agent at 2X final concentration were dispensed into the wells of plastic microdilution trays and sealed and frozen at -70°C until they were used.

Broth microdilution testing was performed in accordance with the guidelines in NCCLS document M27-A2. The inoculum suspension was prepared by the spectrophotometric method obtaining a final inoculum of (2.5 x 1.0) x 10³ cells ml⁻¹. Aliquots of 100 µL yeast inoculum were added to each well of the microdilution trays. The final concentrations of the antifungal agents were 0.015-16 µL for amphotericin B and itraconazole and 0.031-64 µL for fluconazole. The trays were incubated at 35°C and MIC endpoints were read after 48 h of incubation. Drug-free and yeast-free controls were included. Following incubation the MICs of fluconazole and itraconazole were read at the lowest concentration at which a prominent decrease (approximately 50%) in turbidity relative to the growth control well was observed. Amphotericin B MICs were read as 100% of growth inhibition. Analytic control was ensured by testing the NCCLS-recommended strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019. The interpretative criteria for susceptibility were those published by NCCLS. Regarding to amphotericin B [9], we established the breakpoint of ≤ 1µL for susceptibility.

Enzymatic profile

Lipolytic activity was tested in Petri dishes containing 0.67% Yeast Nitrogen Base (YNB) and 2% agar plus 0.5% tween 20 as carbon source. The

tested strains were previously inoculated in sterile distilled water for 24 hours in order to exhaust their endogenous carbon resources. Plates were incubated at 22-25°C for three weeks. Growth of colonies in the tested medium was considered positive for the production of lipase. Production of caseinase (proteinase) was tested in Petri dishes containing 0.67% Yeast Nitrogen Base (YNB) plus 0.5% glucose, 0.5% casein and 2% agar (pH 7). Plates were incubated for 7 days at 22 – 25°C. Evaluation of results was through addition of HCl 1N over the medium. Result was considered positive when there was the appearance of a transparent halo around the yeast inoculum over a whitish background due to the denaturation of the casein.

RESULTS

Yeasts were isolated from 59% of the samples (11 raw milk and 2 cheese samples). Fifty six strains were isolated and identified in the genera *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Pichia*, *Rhodotorula*, *Sporodiobolus*, *Trichosporon*, *Yarrowia* and *Zygoascus* (Table 1). Their counting can also be seen in Table 1. The average total yeast count in raw milk was superior to 2 log UFC.mL⁻¹, while cheese count was superior to 3 log UFC.g⁻¹. Lipolytic activity was present in almost 92% of the isolates, while only 14% had proteolytic activity (data not shown).

Twelve potentially pathogenic ascomycetic isolates were identified by the conventional yeast identification methodology, and correspond to the species *Candida parapsilosis*, *Candida tropicalis* and *Pichia guilliermondii* (Table 1). All of them had their identities confirmed by the HMA assay (Table 2). None of the strains presumptively belonging to potentially pathogenic species were resistant to the antifungal agents tested (Table 3).

DISCUSSION

Raw goat milk samples presented counts (1.51 - 4.41 log UFC.mL⁻¹) similar to the ones found in raw cow milk collected in the same area [32]. Although there is no yeast count standards established for raw milk, these high numbers can influence the diversity of the mycobiota that will develop in the cheese and other milk derivatives. Yeast count in goat creamed cheese was variable but superior to >3 log UFC.g⁻¹. In Cheddar cheese, counting was between 2 and 7 log UFC.g⁻¹ [40], while mould and yeast counting varied

Table 1. Conventional identification and counting of yeasts isolated from raw goat milk and goat cheese.

SPECIES	NUMBER OF ISOLATES	RAW MILK (n=15)											RAW MILK COUNT log UFC/mL	CHEESE (n=10)		CHEESE COUNT log UFC/g
		S1 ^a	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11		S12	S13	
<i>Candida aaseri</i>	1					+							1,51			
<i>Candida catenulata</i>	4	+	+					+		+			2,75		+	3,32
<i>Candida drosophilae-like</i>	1							+					1,51			
<i>Candida glabrosa-like</i>	1				+								2,49			
<i>Candida parapsilosis</i>	7	+		+	+	+			+				2,32	+	+	2,14
<i>Candida sake</i>	1											+	1,51			
<i>Candida sorbophila</i>	1						+						2			
<i>Candida tropicalis</i>	1		+										2,51			
<i>Candida zeylanoides</i>	1				+								1,82			
<i>Candida</i> sp. 1	1	+											1,51			
<i>Candida</i> sp. 2	1	+											2			
<i>Bullera</i> sp. 1	1											+	1,82			
<i>Bullera</i> sp. 2	1											+	2,36			
<i>Cryptococcus curvatus</i>	2			+					+				2,97			
<i>Cryptococcus flavus-like</i>	1								+				1,82			
<i>Cryptococcus humicola</i>	1							+					>2			
<i>Cryptococcus hungaricus</i>	2							+	+				2,94			
<i>Cryptococcus hungaricus-like</i>	1								+				1,82			
<i>Cryptococcus terreus-like</i>	1								+				1,82			
<i>Cryptococcus</i> sp. 1	1		+										1,82			
<i>Cryptococcus</i> sp. 2	1							+					2,9			
<i>Cryptococcus</i> sp. 3	1									+			1,51			
<i>Debaryomyces hansenii</i>	2									+	+		1,81			
<i>Dekkera anomala</i>	1											+				1,51
<i>Dekkera bruxellensis</i>	2				+	+							2,39			
<i>Pichia guilliermondii</i>	4					+	+	+			+		2,07			
<i>Pichia</i> sp.	1		+										1,51			
<i>Rhodotulula glutinis</i>	1													+		1,51
<i>Rhodotulula minuta</i>	1		+										1,51			
<i>Rhodotorula mucilaginosa</i>	1					+							1,82			
<i>Sporidiobolus</i> sp.	1		+										2,33			
<i>Trichosporon ovooides</i>	1						+						2,56			
<i>Trichosporon</i> spp.	2													+	+	1,66
<i>Yarrowia lipolytica</i>	2													+	+	2,52
<i>Zygoascus hellenicus</i>	1						+						1,82			
TOTAL YEAST COUNT / SAMPLE log UFC/mL or g		2.81	2.95	2.79	2.61	3.36	2.66	4.41	3.67	2.94	2.66	1.51		3.26	3.37	

^aPositive samples

Table 2. Molecular confirmation of the identification of strains that presumptively belong to clinically relevant yeast species by means of HMA of the D1/D2 region of 26S rDNA.

SPECIES/STRAIN	SAMPLE CODE	POSITIVE CONTROL ^a	NEGATIVE CONTROL ^b	HMA CONFIRMATION
<i>Candida parapsilosis</i>				
LC04	S1	no heteroduplex	heteroduplex	+
LC13	S3	no heteroduplex	heteroduplex	+
LC20	S4	no heteroduplex	heteroduplex	+
LC24	S5	no heteroduplex	heteroduplex	+
LC48	S8	no heteroduplex	heteroduplex	+
LC66	S12	no heteroduplex	heteroduplex	+
LC85	S13	no heteroduplex	heteroduplex	+
<i>Candida tropicalis</i>				
LC12	S2	no heteroduplex	heteroduplex	+
<i>Pichia guilliermondii</i>				
LC27	S5	no heteroduplex	heteroduplex	+
LC31	S6	no heteroduplex	heteroduplex	+
LC46	S7	no heteroduplex	heteroduplex	+
LC78	S10	no heteroduplex	heteroduplex	+

a – Positive controls were the corresponding type strains. b – Negative controls were *C. parapsilosis* for presumptive *C. tropicalis* strains, and *C. tropicalis* for presumptive *C. parapsilosis* and *P. guilliermondii* strains.

* + identification confirmed

between 4 and 8 log UFC.g⁻¹ in samples of coalho and butter cheese [14]. Yeast counts in raw milk inferior to 4 log CFU/mL may be considered low [15], whereas superior counts may indicate deficiencies in the hygienization, milking and conservation of milk [5].

A high percentage of the isolates presented lipolytic activity (almost 92%), while only 14% were proteolytic. The high production of lipase seems to be common in raw milk isolates [32]. Production of extracellular enzymes may alter the organoleptic characteristics of raw milk [26] and, as microorganisms can survive the thermal treatments, they can cause alterations in the texture and flavor of lactic products during the storage [7,37]. On the other hand, the presence of yeasts may be beneficial, contributing to the manufacture of lactic products and certain cheese types through the interaction with start cultures of lactic acid bacteria and the secondary flora composed by bacteria and other fungi [39].

In spite of the low sampling number, samples of raw goat milk presented a great number of distinct species (at least 32), suggesting a great diversity (Table 1). This was also found in raw cow milk

samples in the same area [32], and seems to be common in this kind of sample. Samples of goat cheese, on the other hand, presented a low number of distinct species. This apparent low yeast diversity is misleading due to the few samples which allowed yeast isolation (only two samples), but each sample had the same diversity (4 species) as found in raw goat milk samples (1 to 6 species per sample).

It is known that the phenotypic characterization of yeasts can lead to errors due to the fact that several species present similarities in their morphologies and biochemical / physiological characteristics used in the conventional identification of yeasts. New molecular techniques are being developed to identify and characterize microorganisms originated from cheese and raw milk [6,8,24]. Recently, Ramos *et al.* (2006) reported a molecular technique for confirmation of the identification of clinically important yeasts belonging to the genus *Candida*. This technique is based on the Heteroduplex Mobility Assay (HMA) of the 26S rDNA D1/D2 region. In a simple explanation, when the D1/D2 PCR products of yeast strains belonging to the same species are mixed, they do not form heteroduplexes in

Table 3. Antifungal susceptibility thresholds of strains that presumptively belong to clinically relevant yeast species isolated from goat milk and cheese.

SPECIES/STRAIN	FLUCONAZOLE CIM µg/mL	ITRACONAZOLE CIM µg/mL	AMPHOTERICIN B CIM µg/mL
<i>Candida parapsilosis</i>			
LC04	0.5	0.03	0.03
LC13	0.5	0.03	0.3
LC20	0.5	0.06	0.015
LC24	0.5	0.06	0.015
LC48	1	0.125	0.06
LC66	1	0.125	0.015
LC85	4	0.125	0.015
<i>Candida tropicalis</i>			
LC12	8	0.0625	0.5
<i>Pichia guilliermondii</i>			
LC27	0.5	0.03	0.015
LC31	1	0.125	0.015
LC46	4	0.125	0.015
LC78	0.5	0.03	0.06

a polyacrilamide gel electrophoresis. When PCR products of strains belonging to different species are mixed, the heteroduplexes are easily detected. Thus, confirmation of the identification of clinically important yeasts is done by separately mixing their PCR products with the products obtained from the type strain of the presumed species and from the other potentially pathogenic *Candida* species, followed by polyacrilamide gel electrophoresis.

The most frequent species isolated from raw goat milk were *C. parapsilosis*, *Candida catenulata* and *P. guilliermondii* (anamorph *Candida guilliermondii*) (Table 1). *C. parapsilosis* and *P. guilliermondii* strains had their identities confirmed by the HMA assay (Table 2). This raises a great concern because these species are considered as potentially pathogenic for humans and other animals [3,29]. Another potentially pathogenic species, *C. tropicalis*, also confirmed by the HMA assay, was isolated from raw milk (Tables 1 and 2). Six out of 11 milk samples with yeast isolation yielded potentially pathogenic yeasts confirmed by molecular methods, but this was not associated with high total yeast numbers. It would be interesting to verify if the goat herd suffers from mastitis caused by

those yeasts. If this is the case, resistance to antifungal agents does not seem to be disseminated, facilitating treatment (Table 3). The other most frequent species in raw goat milk, *Candida catenulata*, although not considered specially pathogenic, seems to be directly related to cases of mycotic mastitis, and was already isolated from milk from animals with mastitis [1,22,33].

The occurrence of these potentially pathogenic yeasts in milk samples has been already reported [19], but there seems to be no difference between the degrees of contamination of the milk collected from animals with and without mastitis [22]. The isolation of yeasts as *C. parapsilosis* and *C. tropicalis* may be related to treatments with antibiotics in animals with mastitis [21]. Most studies concerning animal mastitis are related to cow, and fungi are not considered primary agents, usually being considered environmental contaminants related to poor hygienic condition of the animal [32]. In cows, mycotic mastitis is predominantly caused by yeasts of the genera *Candida*, *Cryptococcus* and *Trichosporon* [18,34]. Richard *et al.* (1980) found *C. tropicalis* and *Candida rugosa* to be the most common species isolated from infected mammary glands of cows in New York and Iowa. An experi-

mental study concerning the potential of *Candida albicans* to cause mastitis in goats proved that there was a sharp fall in milk yield, and *C. albicans* was directly demonstrated in the milk and re-isolated from the milk and udder tissues up to 30th day after inoculation [31].

Yeast species isolated from cheese were identified as *C. parapsilosis* (strains identities confirmed by the HMA assay), *C. catenulata*, *Yarrowia lipolytica*, *Rhodotorula glutinis* and *Trichosporon* spp. (Tables 1 and 2). *C. parapsilosis*, *C. catenulata* and *Trichosporon* sp. were also found in goat milk samples, and are present in raw milk from different species and in several types of cheese [11,12,23]. This suggests that these species may survive the treatments for cheese manufacture and may be spread to the human population, being a question of public health concern.

Rhodotorula glutinis, *R. mucilaginosa* and *R. minuta*, found in goat milk and cheese, are frequently isolated from lactic products, and their presence may be related to environmental contamination during the manufacture of the products [4,41]. Only three strains of the genus *Trichosporon* were isolated. Some species of this genus are described as eventual pathogens of man and animals, and were also isolated from the udders of animals with mastitis [4,38]. The

occurrence of *Trichosporon* spp. in lactic products can mean lack of hygiene [42]. In goat milk, concentration and frequency of this genus was low, opposed to what was found in raw cow milk [32].

In order to rapidly confirm the identification of the strains belonging to potentially pathogenic yeast species, a simplified HMA assay was developed. In this simplified assay, the PCR product corresponding to the D1/D2 region of the 26S rDNA of the milk / cheese isolate was mixed with the amplicon of the corresponding type strain (positive control) and with the amplicon of the type strain of another pathogenic *Candida* species (negative control). This simplified assay was able to confirm the identity of all strains that presumptively belonged to potentially pathogenic *Candida* species, and may be used each time there is a presumptive non-molecular identification, being able to rapidly confirm or discard this identification.

In summary, the presence of potentially pathogenic yeasts in goat raw milk and creamed cheese collected in South Brazil raises concern. The health of the herd should be evaluated, as well as the potential of spread of these yeasts through goat milk and its derivatives. More studies are necessary to evaluate the real significance of the isolation of these clinically relevant yeasts.

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