



Identification and enzymatic profile of yeasts isolated from artisanal cheese in Southern Brazil

Identificação e perfil enzimático de leveduras isoladas de queijo artesanal no sul do Brasil

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ABSTRACT

Yeasts may contribute positively to the characteristic taste and flavour development during cheese ripening or cause deterioration of the product. In this study we report the presence of yeasts in 59 samples of artisanal cheeses (43 samples of colonial cheese, 5 of ricotta, 9 of provolone, and 2 of Caccio Cavallo) marketed at stands in the coastal area of Rio Grande do Sul, collected between November 2004 and June 2005. Yeast counts were between 10^4 and 10^7 UFC/g. One hundred and ten strains were isolated from 30 representative cheese samples. Of these, 97 isolates had ascomycetic affinity, and only 13 were basidiomycetes. The isolates belong to the genera *Candida*, *Debaryomyces*, *Dekkera*, *Dipodascus*, *Galactomyces*, *Kluyveromyces*, *Kodamaea*, *Pichia*, *Rhodospiridium*, *Saccharomyces*, *Schizoblastosporion*, *Sporidiobolus*, *Torulaspora*, *Trichosporon*, *Yarrowia*, and *Zygosaccharomyces*. The predominant species found were *Yarrowia lipolytica*, *Debaryomyces hansenii*, and *Candida zeylanoides*. More than 56% of the strains were lipolytic, while almost 13% were caseinolytic, and approximately 31% were gelatinolytic. The presence of potentially pathogenic and/or deteriorating yeasts emphasizes the need for a better hygienic control of cheese manufacturing and storage. The ability these microorganisms have to produce proteolytic and lipolytic enzymes enhance the potential risk of cheese deterioration during storage.

Key words: yeasts, cheese, enzymatic activity, protease, lipase.

RESUMO

Leveduras podem contribuir positivamente para o desenvolvimento do sabor e flavor característicos durante a maturação do queijo ou podem causar deterioração do produto. Neste estudo, nós reportamos a presença de leveduras em 59 amostras de queijo artesanal (43 amostras de queijo colonial, 5 de ricota, 9 de provolone e 2 de Caccio Cavallo) vendidas em bancas na área costeira do Rio Grande do Sul, coletadas entre novembro de 2004 e junho de 2005. A contagem de leveduras ficou entre 10^4 e 10^7 CFU/g. Cento e dez cepas foram isoladas a partir de 30 amostras representativas de queijo. Noventa e sete isolados tiveram afinidade ascomicética e apenas 13 foram basidiomicetos. Os isolados pertencem aos gêneros *Candida*, *Debaryomyces*, *Dekkera*, *Dipodascus*, *Galactomyces*, *Kluyveromyces*, *Kodamaea*, *Pichia*, *Rhodospiridium*, *Saccharomyces*, *Schizoblastosporion*, *Sporidiobolus*, *Torulaspora*, *Trichosporon*, *Yarrowia* e *Zygosaccharomyces*. As espécies predominantes encontradas foram *Yarrowia lipolytica*, *Debaryomyces hansenii* e *Candida zeylanoides*. Mais de 56% das cepas foram lipolíticas, enquanto cerca de 13% foram caseinolíticas e aproximadamente 31% foram gelatinolíticas. A presença de leveduras potencialmente patogênicas e/ou deterioradoras enfatiza a necessidade de um melhor controle higiênico da manufatura e estocagem do queijo. A habilidade desses microrganismos de produzirem enzimas proteolíticas e lipolíticas aumenta o risco potencial de deterioração do queijo durante a estocagem.

Descritores: leveduras, queijo, atividade enzimática, protease, lipase.

INTRODUCTION

The presence of yeasts in cheese is largely known, and these microorganisms may be originated from the unpasteurized milk or the environment where the cheese is manufactured, mainly the equipment and brines [11,22]. Some yeast species, as *Yarrowia lipolytica* and *Geotrichum candidum*, produce extracellular enzymes in enough amounts to modify the organoleptic characteristics of cheese and to contribute directly to its ripening [6,12,13,25]. Other species produce growth factors, such as amino acids and vitamins, which support the growth of other microorganisms that contribute to cheese maturation [10]. There are few reports of pathogenic yeasts associated to cheese, but Minervini *et al.* [16] isolated *Candida albicans* from a stracciatella cheese sample, and called attention to the fact that high yeast counts may represent a risk for human health. On the other hand, isolation of yeasts with potential to deteriorate cheese is relatively common, since yeasts that participate in cheese maturation can, depending on several factors, cause its deterioration [7,24].

In this study we report the diversity of yeasts in samples of artisanal cheese collected at the coastal area of Rio Grande do Sul, and their capacity of producing extracellular enzymes. Our aim is to verify the presence of potentially pathogenic and/or deteriorating yeasts in the samples in order to evaluate the impact for the consumer's health and for the shelf life of the products.

MATERIALS AND METHODS

Cheese samples

This study was accomplished with 59 samples of artisanal cheeses (43 samples of colonial cheese, 5 of ricotta, 9 of provolone, and 2 of Caccio Cavallo) marketed at stands in the coastal area of Rio Grande do Sul, collected between November 2004 and June 2005. After removal of pieces of approximately 1 cm thickness from the external part of each cheese, 25 g of each sample were added to 225 mL of peptone water, and homogenized in a Stomacher for 90 seconds.

Isolation and identification of yeasts

Aliquots of 0.1mL from serial dilutions were spread in duplicate on acidified YM agar medium (1% glucose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 2% agar, 400 mg/L of chloranphenicol, pH 4.0) or acidified YEPG agar medium (0.5% yeast extract, 2% glucose, 1% peptone, 2% agar, 400 mg/L of chloranphenicol, pH 4.0). After incubation at 22-25°C

for 3-5 days, yeast colonies were counted and the results were expressed as UFC/g of cheese sample. Representative colonies of each morphological type obtained from 30 cheese samples were isolated and purified in Petri dishes containing YEPG medium. The strains were maintained in GYMP medium (0.5% glucose, 2% malt extract, 0.5% yeast extract, 0.2% monobasic sodium phosphate, 2% agar) slants covered with a layer of sterile mineral oil, and kept in the refrigerator. The isolates were phenotypically characterized by standard tests [3,26]. Identification was performed according to [3] and the computer program YEASTCOMPARE [5].

Enzyme assay

For determination of lipolytic activity, the strains were previously grown on YEPG agar at 22-25°C for 24-48h, diluted in sterile distilled water to about 4×10^5 cells/mL, incubated for another 24h, and used to inoculate the solidified agar surface of the suitable medium. Lipase production medium contained 0.67% yeast nitrogen base, 2% agar, and 0.5% tween 20 (polyoxyethylene sorbitan monolaureate 20) as the sole carbon source. The strains were incubated at 22-25°C for 7 days and the enzymatic activity was indicated by yeast growth onto the plates. Protease production was tested by inoculation on Casein agar (0.67% yeast nitrogen base, 0.5% glucose, 0.5% casein and 2% agar), adjusted to pH 7.0 with 5N KOH, and incubation at 22-25°C for 7 days. After precipitation with 1M HCl, protease production was indicated by the presence of a clear zone around the colonies [19]. The hydrolysis of gelatin was also used to verify the protease production. Yeasts were inoculated in tubes containing malt-gelatin (10% extract of malt and 12% gelatin), and incubated of 22-25°C for 21 days. Protease activity was verified through the hydrolysis of the gelatin, and consequent liquefaction of the medium [1].

RESULTS

Yeast counts were between 10^4 and 10^7 UFC/g (data not shown). One hundred and ten strains were isolated from 30 samples of colonial, provolone, ricotta and Caccio Cavallo cheese. Of these, 97 isolates had ascomycetic affinity, and only 13 were basidiomycetes. The identified isolates belong to the genera *Candida*, *Debaryomyces*, *Dekkera*, *Dipodascus*, *Galactomyces*, *Kluyveromyces*, *Kodamaea*, *Pichia*, *Rhodospiridium*, *Saccharomyces*, *Schizoblastosporion*, *Sporidiobolus*, *Torulaspora*, *Trichosporon*, *Yarrowia*, and *Zygosaccharomyces* (Table 1). The lipolytic and

proteolytic profile of the strains can be found on Table 2. More than 56% of the strains were lipolytic. Proteolytic capacity was verified through production of caseinase and gelatinase. Almost 13% of the strains were caseinolytic, while approximately 31% were gelatinolytic.

DISCUSSION

Several authors have reported a broad range of yeast numbers (10^1 to 10^9 UFC/g) in cheese, depending on the cheese type and producer [2,8,23]. In Brazil, Souza *et al.* [18], studying the serrano cheese produced in Rio Grande do Sul, found yeast counts between 10^3 and 10^6

Table 1. Frequencies of yeasts isolated from 30 artisanal cheese samples from the coastal area of Rio Grande do Sul, collected between November 2004 and June 2005.

Yeasts	Total number of isolates	Number of isolates per cheese type			
		Colonial cheese n=20	Ricotta n=4	Provolone n=4	Caccio cavallo n=2
<i>Candida catenulata</i>	3	3	-	-	-
<i>Candida haemulonii</i> -like	1	-	-	1	-
<i>Candida intermedia</i>	1	-	1	-	-
<i>Candida lusitanae</i>	4	4	-	-	-
<i>Candida maltosa</i>	2	2	-	-	-
<i>Candida maltosa</i> -like	1	1	-	-	-
<i>Candida parapsilosis</i>	2	-	1	1	-
<i>Candida pseudogloeobosa</i>	1	1	-	-	-
<i>Candida restingae</i>	1	1	-	-	-
<i>Candida rugosa</i>	3	2	-	1	-
<i>Candida sake</i>	3	2	1	-	-
<i>Candida sake</i> -like	1	1	-	-	-
<i>Candida sorbophila</i> -like	1	1	-	-	-
<i>Candida vinaria</i> -like	2	1	1	-	-
<i>Candida zeylanoides</i>	9	7	1	-	1
<i>Candida zeylanoides</i> -like	1	-	1	-	-
<i>Candida</i> spp.	7	3	3	1	-
<i>Debaryomyces hansenii</i>	11	6	2	2	1
<i>Debaryomyces maramus</i>	1	1	-	-	-
<i>Dekkera anomala</i>	1	1	-	-	-
<i>Dekkera bruxelensis</i>	1	1	-	-	-
<i>Dipodascus</i> sp.	1	1	-	-	-
<i>Galactomyces geotrichum</i>	3	3	-	-	-
<i>Kluyveromyces lactis</i>	6	5	1	-	-
<i>Kluyveromyces marxianus</i>	3	2	-	1	-
<i>Kodamaea ohmeri</i>	1	-	1	-	-
<i>Pichia guilliermondii</i>	1	1	-	-	-
<i>Pichia rabaulensis</i> -like	1	1	-	-	-
<i>Pichia scolytii</i> ?	1	1	-	-	-
<i>Rhodospidium toruloides</i>	3	1	2	-	-

Table 1. (cont.)

Yeasts	Total number of isolates	Number of isolates per cheese type			
		Colonial cheese n=20	Ricotta n=4	Provolone n=4	Caccio cavallo n=2
<i>Saccharomyces cerevisiae</i>	2	2	-	-	-
<i>Saccharomyces unisporus</i>	1	1	-	-	-
<i>Saccharomycopsis crataegensis</i>	1	1	-	-	-
<i>Schizoblastosporion starkeyi-henricii</i>	1	-	-	1	-
<i>Schizoblastosporion</i> sp.	1	-	-	1	-
<i>Sporidiobolus pararoseus</i>	1	-	-	1	-
<i>Torulaspora delbrueckii</i>	1	-	-	-	1
<i>Trichosporon inkin</i>	4	2	1	1	-
<i>Trichosporon loubieri</i>	4	3	-	1	-
<i>Trichosporon ovoides</i>	1	1	-	-	-
<i>Yarrowia lipolytica</i>	15	11	2	1	1
<i>Zygosaccharomyces bisporus</i>	1	1	-	-	-

n = number of samples.

Table 2. Production of proteases (caseinase and gelatinase) and lipase by yeasts isolated from 30 artisanal cheese samples from the coastal area of Rio Grande do Sul, collected between November 2004 and June 2005.

Species	Caseinase	Gelatinase	Lipase
<i>Candida catenulata</i>	1 ^a	2	2
<i>Candida haemulonii</i> -like	- ^b	-	1
<i>Candida intermedia</i>	-	-	1
<i>Candida lusitanae</i>	-	-	2
<i>Candida maltosa</i>	-	2	2
<i>Candida maltosa</i> -like	-	1	-
<i>Candida parapsilosis</i>	2	-	1
<i>Candida pseudogloeobosa</i>	-	-	-
<i>Candida restingae</i>	-	-	1
<i>Candida rugosa</i>	1	1	1
<i>Candida sake</i>	-	1	3
<i>Candida sake</i> -like	1	-	1
<i>Candida sorbophila</i> -like	-	-	-

UFC/g, while Feitosa *et al.* [9] reported yeast counts between 10^4 and 10^8 UFC/g in coalho and butter cheese from Rio Grande do Norte, and Borelli [4] found 10^2 to 10^7 UFC/g in the curd and Canastra cheese artisanally produced in Minas Gerais. We have found yeast counts between 10^4 and 10^7 UFC/g, a range that is very similar to those reported by other authors. Thirty nine out of 43 colonial cheese samples had counts over 10^5 UFC/g, including 27 samples with counts over 10^6 UFC/g (data not shown). There is a concern about this high count because yeast numbers higher than 10^6 - 10^7 UFC/g can deteriorate the product [10]. Distribution of yeast counts within the 10^4 to 10^7 UFC/g range was even for the other cheese sampled.

The predominant species found in the analyzed cheese samples were *Yarrowia lipolytica*, *Debaryomyces hansenii*, and *Candida zeylanoides*, although *C. zeylanoides* was not isolated from provolone cheese, probably due to the small sample size. These

species are commonly isolated from cheese and other food items [2,4,8,17]. *Y. lipolytica* has already been proven to produce enough extracellular enzymes to modify the organoleptic characteristics of cheese during its maturation [6,12,13,25]. *D. hansenii* is commonly associated with cheese manufacturing, and its presence is probably due to its high salt tolerance, among other factors [21]. The high frequency of *C. zeylanoides*, which is often associated to food deterioration, may be an

indicative of the poor sanitary conditions these artisanal cheese are being produced and stored prior to sale.

Trichosporon inkin was found in three cheese types. *T. inkin* belongs to a group of potentially pathogenic yeasts commonly isolated from cheese and cheese factories. Apart from *T. inkin*, other clinically relevant yeasts have been isolated, although in low numbers. *Candida lusitanae* was found in four and *Pichia guilliermondii* in one out of 20 samples of colonial cheese, and

Candida parapsilosis in one out of four samples each of provolone and ricotta cheese. The isolation of these clinically relevant yeasts may reflect the poor hygienic conditions the cheeses are produced, and the fact that these yeasts are capable of surviving the conditions of cheese manufacturing. They are also commonly found in raw milk [15]. So, it is probable that their source is the milk used for manufacturing the cheese. *Trichosporon loubieri* and *Trichosporon ovoides* were also isolated from few samples of colonial and/or provolone cheese. *T. loubieri* has been isolated from dairies, cow with mastitis and mastitic milk [3], and its source is probably also the milk used to produce cheese. *T. ovoides* is associated with cases of white piedra and skin [3], and is a probable contaminant from human origin, emphasizing the poor hygienic conditions of cheese production and storage.

The isolation of yeasts with potential to deteriorate cheese is very common. Fadda *et al.* [7] reported the association of altered packages of feta cheese with *Dekkera anomala*. This species is capable of a strong fermentation of lactose, producing gas and altering the package. We have found *D. anomala* and *Dekkera bruxelensis* in samples of colonial cheese, but they do not seem to be common. We have isolated *Torulaspora delbrueckii* from one out of two samples of Caccio Cavallo cheese. As this yeast is also considered a potentially deteriorating microorganism, this isolation may be of concern.

Few strains with basidiomycetic affinity other than *Trichosporon* were found. *Rhodospiridium toruloides* was isolated from one sample of colonial cheese and two of ricotta, while *Sporidiobolus pararoseus*

Table 2. (cont.)

Species	Caseinase	Gelatinase	Lipase
<i>Candida sorbophila</i> -like	-	-	-
<i>Candida vinaria</i> -like	-	1	1
<i>Candida zeylanoides</i>	1	-	4
<i>Candida zeylanoides</i> -like	-	-	-
<i>Candida</i> spp.	1	3	5
<i>Debaryomyces hansenii</i>	-	-	4
<i>Debaryomyces maramus</i>	-	-	1
<i>Dekkera anomala</i>	-	-	-
<i>Dekkera bruxelensis</i>	-	-	1
<i>Dipodascus</i> sp.	-	1	1
<i>Galactomyces geotrichum</i>	-	3	-
<i>Kluyveromyces lactis</i>	-	-	2
<i>Kluyveromyces marxianus</i>	-	-	1
<i>Kodamaea ohmeri</i>	-	-	-
<i>Pichia guilliermondii</i>	-	-	1
<i>Pichia rabaulensis</i> -like	-	-	1
<i>Pichia scolytii</i> ?	-	1	1
<i>Rhodospiridium toruloides</i>	1	-	3
<i>Saccharomyces cerevisiae</i>	-	-	1
<i>Saccharomyces unisporus</i>	-	-	-
<i>Saccharomycopsis crataegensis</i>	-	1	1
<i>Schizoblastosporion starkeyi-henricii</i>	-	-	-
<i>Schizoblastosporion</i> sp.	1	1	-
<i>Sporidiobolus pararoseus</i>	-	-	1
<i>Torulaspora delbrueckii</i>	1	-	-
<i>Trichosporon inkin</i>	-	2	3
<i>Trichosporon loubieri</i>	-	1	3
<i>Trichosporon ovoides</i>	-	-	1
<i>Yarrowia lipolytica</i>	4	13	11
<i>Zygosaccharomyces bisporus</i>	-	-	-

^anumber of positive strains; ^bnegative result.

was found in one sample of provolone cheese. This low frequency of basidiomycetic yeasts may mean that they are not common inhabitants of cheese samples, but instead they are probably airborne contaminants.

Colonial and ricotta cheese can be considered moisty, while provolone and Caccio Cavallo are dry cheese. Although the differences in sample size among the analyzed cheese can limit the conclusions, it is worth noting that *Candida haemulonii*-like, *Schizoblastosporium starkeyi-henricii*, *Schizoblastosporium* sp. and *S. pararoseus* were only found in dry cheese in spite of the low sample number (6 samples). At present, it is not possible to infer if the three former species are airborne contaminants as it is believed for *S. pararoseus*. Moisty cheese are more represented in our samples (20 samples of colonial and 4 of ricotta). The isolation of a species from only colonial cheese is not very informative, as it can be biased by the sample number, but isolation from both colonial and ricotta of a species not present in dry cheeses may be meaningful. It is the case of *K. lactis*, *Candida vinaria*-like and *R. toruloides*. The latter two species were found in few samples, and can represent eventual contaminants, but isolation of *K. lactis* only from moisty cheese is noteworthy. *K. lactis* is a species commonly isolated from cheese, and its presence in dairy products is usually explained by its ability to assimilate and ferment the lactose present in milk [8]. It would be interesting to test if this species is able to survive and reproduce in the harsh conditions of dry cheese.

Most of the reports in the literature deal with proteolytic and lipolytic activities of few selected strains isolated from cheese, thus preventing comparisons with our results. The low percentage of proteolytic strains

in our samples may reflect the fact that protease production was tested only in neutral conditions. pH variations during protease production may influence the results [20], so it would be interesting to perform the tests also in more acidic conditions. Strains were more gelatinolytic than caseinolytic. As casein is the main milk protein, it seems that the proteolytic capacity of the isolates has a low influence over cheese protein content. More than 56% of our isolates were lipolytic, although some of them were weak lipase producers (data not shown). Borelli [4] found only 3.2% of lipolytic strains in Canastra cheese in Minas Gerais. This discrepancy is probably due to the methodology these authors employed for verifying lipolytic activity, since they used tributyrin instead of tween 20 as the substrate for lipase production. Differences in the substrate used in lipase assays may detect enzymes with different specificities [14], explaining these results.

CONCLUSION

In summary, the presence of potentially pathogenic and/or deteriorating yeasts in cheese samples collected in the coastal area of Rio Grande do Sul emphasizes the need for a better hygienic control of cheese manufacturing and storage. The ability these microorganisms have to produce proteolytic and lipolytic enzymes enhance the potential risk of cheese deterioration during storage.

Acknowledgements. The authors thank Dr. Marisa da Costa and Cristina Zaffari (Msc.) for kindly providing the cheese samples. This work was supported by PROPESQ/UFRGS (financial support n° 237). M.F. Landell and C.C. Hartfelder received fellowships from CNPq and FAPERGS, respectively.

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