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ORIGINAL PAPER

# Yeast populations associated with the artisanal cheese produced in the region of Serra da Canastra, Brazil

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**Abstract** The aim of this work was to describe the yeast populations present during the manufacturing of Minas cheese of the region of Serra da Canastra, Minas Gerais state, Brazil. Canastra cheese is produced from raw cow's milk at the farmhouse level using artisanal procedures and natural whey cultures as starters. Samples from 10 farms were studied, and they included: raw milk, natural starter, cheese curd before salting and cheese after 5 days of ripening. The most frequent yeasts in whey, curd and cheese were *Debaryomyces hansenii*, *Kluyveromyces lactis, Kodamaea ohmeri* and *Torulaspora delbrueckii*. Many yeast isolates were able to produce proteases, lipases and $\beta$-galactosidades. Production of these enzymes by yeasts in the cheese would contribute to the development of the characteristic flavor and smell during the ripening process.

**Keywords** Yeasts · Artisanal Canastra cheese · Natural starter · Proteases · Lipases · $\beta$-galactosidades

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## Introduction

Minas cheese is one of the most appreciated dairy products in Brazil. It originates from state of Minas Gerais and represents about 42% of Brazilian cheese production. It is a semi-hard cheese, produced with pasteurized cow milk, with a pale cream color, yellowish rind, cylindrical shape and homogeneous texture. The flavor is described as piquant and mildly acidic. Typical weights range from 800 g to 1200 g (Brasil 1998; INDI 2002). Canastra cheese is the denomination of origin of a variety of Minas cheese produced with raw cow milk in the region of Serra da Canastra in Minas Gerais state. This cheese has been manufactured in a traditional empirical manner for more than 200 years in the region; it has, thus, a great social and cultural value, as well as economic significance. The region produces about 375.5 tonnes of cheese per month. Besides being the main economic activity of many families of the region, artisanal manufacturing of Canastra cheese also reflects a cultural heritage from Brazil passed on from generation to generation. Canastra cheese technology was introduced into the region by Portuguese immigrants in the late eighteenth century following the techniques for production of Serra da Estrela cheese. Later, this technology was modified and adapted to environmental conditions of this region. Canastra cheese is traditionally made with raw cow's milk employing natural whey cultures as starters (indigenous lactic acid bacteria) and commercial rennet. The natural starter (''pingo'') is produced by dropping whey from previous cheese covered with salt, and the microbiota is composed by species of *Lactobacillus*, *Lactococcus* and *Streptococcus*, with counts of approximately 8 log c.f.u. $ml^{-1}$ (Borelli and Rosa, unpublished data). Cheesemaking almost always take place in a room adjacent to the cow barn where the raw milk is filtered from the

milking bucket into the cheese vat. Milk is coagulated by adding commercial calf rennet and 0.2–0.5 l of natural starter culture per 100 l of milk. The native microflora present in the raw milk, ''pingo'' and in the cheese-making equipments provided the bacteria that produced acid during the cheese-making process. One hour after the addition of the rennet and the ''pingo'', the coagulum is cut and transferred to plastic moulds where the whey is removed by hand pressing. The cheese is covered with salt for 6 h, inverted, then salted again for 18 h. Salt is removed and cheeses are then ripened in wooden shelves without temperature or humidity control, being turned periodically. The ripening period lasts between 3 days and 10 days. Canastra cheese exhibits a characteristic flavor appreciated by consumers all over Brazil.

Yeasts have emerged as significant organisms in the cheese maturation process, although their precise role is not understood. Many studies show the occurrence of yeasts linked to ripening processes in many different cheeses (Welthagen and Viljoen 1999; Pereira-Dias et al. 2000; Ferreira and Viljoen 2003; Fadda et al. 2004). Yeasts, as *Debaryomyces hansenii, Kluyveromyces lactis*, and *Yarrowia lipolytica*, may contribute positively to flavor development during the stage of ripening (van den Tempel and Jakobsen 2000; Romano et al. 2001). The yeasts could assist the starter cultures in cheeses by proteolytic activity, lipolytic activity, and possibly participate in the maturation, including the formation of aroma components (Jakobsen and Narvhus 1996; Tornadijo et al. 1998; Welthagen and Viljoen 1999; Suzzi et al. 2001). In contrast, yeasts may also lead to product spoilage. Typical defects caused by spoilage yeasts are gas production, yeasty flavor and other off-flavors, discolorations and changes of cheese texture (Fleet 1990; Jakobsen and Narvhus 1996; Welthagen and Viljoen 1999; Pereira-Dias et al. 2000). In Danish feta cheese, swelling of samples was caused by *Torulaspora delbrueckii*, and high concentrations of *D. hansenii* and *Y. lipolytica* were responsible for a strong yeast smell and unwanted texture of the cheese due to degradation of fat, respectively (Westall and Filtenborg 1998). There is little information available on the microbiological characteristics of artisanal Brazilian cheeses (Souza et al. 2003). The aim of this study is to characterize the yeast populations during cheesemaking and early ripening of the artisanal cheese produced in region of Serra da Canastra, Brazil.

## Material and methods

### Sample collection

Samples were obtained from ten different farms (A to J) in the city of São Roque de Minas, State of Minas Gerais, Brazil, in December 1999 and February 2000. Raw milk, whey, cheese curd before salting and cheese after 5 days of ripening at room temperature from each farm were sampled aseptically, and transported to the laboratory under refrigeration for microbiological analyses.

### Microbiological analysis—Milk, natural starter, curdle and cheese samples

Aliquots of 10 ml of milk and natural starter were diluted in 90 ml sterile 0.1% buffered peptone water before inoculation. For curd and cheese 25 g portions were homogenized with 225 ml of 0.1% buffered peptone water in a Stomacher 400 Lab Blender (London, UK) for 1 min and decimal dilutions were prepared there from using the same diluents. For determination of yeast populations, aliquots (0.1 ml) of appropriate decimal dilutions were spread on yeast extract-malt extract agar (YMA—1% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract and 2% agar) containing 200 mg chloramphenicol $l^{-1}$. Plates were incubated at 25°C for 5 days. After growth, the different yeast colonies were counted, isolated and maintained on YMA slants or in liquid nitrogen. Yeasts were characterized phenotypically by methods currently used in yeast taxonomy (Yarrow 1998). The physiological tests used were fermentation of D-glucose, D-galactose, sucrose, maltose, lactose and raffinose; growth on the D-glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, *N*-acetyl-D-glucosamine, methanol, ethanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, xylitol, salicin, D-gluconate, DL-lactate, succinate, citrate, inositol, hexadecane, acetone, 2-propanol and ethyl acetate as carbon sources; growth on nitrate, nitrite, L-lysine, ethylamine and cadaverine as nitrogen sources; growth at 30, 37, 40°C; growth on media with 50% of glucose, and with 10% of NaCl; tolerance to 1% of acetic acid; formation of extracellular amyloid compounds; Diazonium Blue B color reaction, and resistance to 0.01% and 0.1% of cycloheximide. Identities were verified using the keys described by Kurtzman and Fell (1998).

Yeasts isolates of uncertain identify were confirmed by sequencing the D1/D2 variable domains of the large subunit rDNA. D1/D2 divergent domains were amplified by PCR as described by Lachance et al. (1999) using the primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′). The amplified DNA was concentrated and cleaned on WizardSV columns (Promega, USA), and sequenced in a MegaBACE™ 1000 automated sequencing system

(Amersham Biosciences, USA). The sequences were edited with the program DNAMAN, version 4.1 (Lynnon Bio-Soft, Vaudreuil, QC, Canada). Existing sequences for other yeasts were retrieved from GenBank.

Lipolytic, proteolytic and $\beta$-galactosidase activities of yeasts

Screening for lipase production was tested as the ability of yeast strains to hydrolyze 2% emulsion of olive oil in polyvinyl alcohol according to Watanabe et al. (1977). Extracellular proteolytic production was tested by inoculating the yeast strains in casein agar adjusted to pH 7.0 by adding 5 M KOH, and to pH 5.0 by adding 1 M HCl (Abranches et al. 1997); plates were incubated at 22°C for 7 days. Proteolytic activity was detected as the presence of a clear zone around the colony after precipitation with 1 M HCl. Gelatin hydrolysis was also used to detect protease production. Yeasts growing in tubes containing malt extract-gelatin-agar medium were incubated at 22°C for 7, 14 or 21 days (Kurtzman and Fell 1998). Gelatin hydrolysis corresponded to liquefaction of the medium. Yeast strains were tested for production of ($\beta$-galactosidase in Yeast Nitrogen Base (Difco, USA) in which lactose was added as the sole carbon source (Kurtzman and Fell 1998). Positive growth was considered as the ability to produce the enzyme.

## Results and discussion

Yeast populations were low in milk samples (Table 1). Samples of natural starter, curd and cheese had counts between 1.7 log c.f.u. $g^{-1}$ or $ml^{-1}$ and 7.9 log c.f.u. $g^{-1}$ or $ml^{-1}$ of each sample. Yeast counts in Canastra cheese were very wide. These differences could be associated with some factors, such as concentration of NaCl, temperature, oxygen availability, substrate availability, and influences of other microorganisms (Addis et al. 2001). The growth of yeasts in milk and dairy products is associated with the ability to assimilate and ferment lactose, to assimilate citric and lactic acids, to produce lipases and extracellular proteases and to tolerate high salt content (Fleet 1990; Fadda et al. 2004). In our study only *Candida zeylanoides* and

**Table 1** Average of populations of the yeast species isolated during the production of the Canastra cheese (log c.f.u. $g^{-1}$ or $ml^{-1}$)

| Species | Raw milk ($n$ = 10)[a] | Natural starter ($n$ = 10) | Curd ($n$ = 10) | Cheese ($n$ = 10) |
|---|---|---|---|---|
| *Candida catenulata*[b] | — | — | 4.2 (1)[c] | 7.8 (5) |
| *C. ethanolica*[b] | — | 2.3 (1) | 2.8 (1) | — |
| *C. galacta* | — | — | — | 5.8 (1) |
| *C. humilis* | — | — | — | 7.2 (1) |
| *C. magnoliae* | — | — | — | 6.9 (1) |
| *C. parapsilosis*[b] | — | 4.3 (1) | — | 5.2 (2) |
| *C. pseudolambica*[b] | — | 3.7 (1) | — | — |
| *C. rugosa* | — | 4.2 (1) | 3.7 (1) | 5.6 (1) |
| *C. silvae*[b] | — | — | 3.3 (1) | — |
| *C. zeylanoides* | 2.0 (1) | 2.7 (1) | — | — |
| *Candida* sp.1 | — | 3.4 (1) | — | — |
| *Candida* sp.2 | — | — | — | 7.4 (1) |
| *Candida* sp.3 | — | 3.9 (1) | — | — |
| *Candida* sp.4 | — | — | 3.6 (1) | — |
| *Cryptococcus albidus* | — | 1.7 (1) | — | — |
| *C. flavus* | — | 3.0 (1) | — | — |
| *C. terreus* | — | — | 3.5 (1) | — |
| *Debaryomyces hansenii*[b] | — | 6.9 (5) | — | 7.8 (6) |
| *Issatchenkia orientalis*[b] | — | — | 2.3 (1) | — |
| *Kodamaea ohmeri*[b] | — | 3.9 (5) | 2.6 (3) | 6.8 (4) |
| *Kluyveromyces lactis*[b] | — | 4.3 (4) | 4.6 (3) | 6.7 (2) |
| *Pichia membranifaciens* | — | — | 2.4 (1) | — |
| *P. subpelliculosa* | — | — | — | 7.4 (1) |
| *Rhodotorula glutinis* | — | — | 1.7 (1) | — |
| *R. mucilaginosa* | — | 4.2 (1) | 1.7 (1) | — |
| *Saccharomyces cerevisiae* | — | — | — | 3.4 (1) |
| *S. exiguus* | — | — | — | 6.3 (1) |
| *Torulaspora delbrueckii*[b] | — | 3.8 (7) | 3.3 (1) | 7.9 (8) |
| *Zygosaccharomyces rouxii* | 2.6 (1) | 3.2 (1) | 3.2 (1) | 7.1 (2) |

[a] $n$ = number of samples

[b] Species identity confirmed by sequencing of D1/D2 domains of the large subunit of rDNA

[c] Number in parenthesis represents the number of samples that the yeast species was isolated

*Zygosaccharomyces rouxii* were isolated from raw milk in populations of 2.0 and 2.6 log c.f.u. $ml^{-1}$, respectively. These yeasts are usually found to be contaminants of raw and pasteurized milk in lower counts as compared to bacterial numbers (Roostita and Fleet 1996). The low yeast numbers in raw milk may be due to competitive utilization of growth substrates, especially by the faster growing bacteria, or inhibition by metabolites excreted by these microorganisms (Viljoen 2001).

Various authors have shown that prevalent yeasts typically associated with cheeses are *D. hansenii*, *K. marxianus, Yarrowia lipolytica* and several *Candida* species (Pereira-Dias et al. 2000; Viljoen 2001; Fadda et al. 2004). In our study, yeast counts in the curd and cheese varied from 1.7 log c.f.u. $g^{-1}$ to 7.9 log c.f.u. $g^{-1}$. *K. ohmeri* and *Kluyveromyces lactis* were the prevalent yeasts in curd samples, and *C. catenulata, D. hansenii* and *T. delbrueckii* in cheese after 5 days of ripening (Table 1). These three species occurred at numbers of about 7.0 c.f.u. $g^{-1}$, and probably play an important part of the natural flora of this cheese. These species have been isolated from different cheeses produced in other countries (Corsetti et al. 2001; Fadda et al. 2004).

In our work, *T. delbrueckii*, *D. hansenii* and *K. ohmeri* were also frequently isolated from natural starter samples. The first species occurred in seven of ten samples of natural starter at an average of 3.8 log c.f.u. $g^{-1}$. Populations of *D. hansenii* and *K. ohmeri* exceeded 6.0 and 3.0 log c.f.u. $ml^{-1}$, respectively. Westall and Filtenborg (1998) reported *T. delbrueckii* to be a major part of the yeast flora in Danish feta cheese. This fermenting yeast was found to cause swelling of the cans. However, the role of this species in the Canastra cheese needs to be determined. *D. hansenii* is a typical foodborne yeast frequently associated with dairy products and is often the prevalent yeast in such systems. Ferreira and Viljoen (2003) suggested to incorporate *D. hansenii* together with *Y. lypolytica* as part of the starter culture to make Cheddar cheese. However, *D. hansenii* have been associated with spoilage of cheese and high counts of this yeast resulted in a strong yeast smell (Westall and Filtenborg 1998).

The occurrence of *K. ohmeri* in Canastra cheese is noteworthy. This is the first report of isolation of *K. ohmeri* associated with cheese manufacturing. This yeast was isolated from five natural starter samples with counts of *ca.* 3.9 log c.f.u. $ml^{-1}$, and from four cheeses with counts of *ca.* 6.8 log c.f.u. $g^{-1}$. Three curdle samples had counts of *ca*. 2.6 log c.f.u. $g^{-1}$. These results suggest that this yeast is part of natural starter microbiota in many of the farms. *K. ohmeri* was initially isolated from cucumber brine and is known in the food industry for its fermentation properties in pickles, salt brines, and fruit spoilage (Kurtzman and Fell 1998). This yeast is frequently associated with flowers and insects but its distribution in nature is not well understood (Rosa et al. 2003). Han et al. (2004) reported human infections by *K. ohmeri* in patients with pre-existing conditions, and in those cases, this species could be considered as an opportunistic pathogen. However, the role of *K. ohmeri* strains in Canastra cheese manufacturing needs to be determined.

The presence of yeasts on the cheese surface is caused mainly by contamination of raw milk, air, clothes, hands, apparatus and equipment used during the production and ripening processes (Elikases-Lechner and Ginzinger 1995;

**Table 2** Production of protease, lipase and $\beta$-galactosidase by the yeast species isolated

| Species | Number of isolates | Number of enzyme-producing isolates | | |
|---|---|---|---|---|
| | | Protease | Lipase | $\beta$-galactosidase |
| *Candida catenulata* | 10 | 6 | — | — |
| *C. parapsilosis* | 3 | 1 | — | — |
| *Candida* sp.1 | 1 | — | — | 1 |
| *Candida* sp.2 | 1 | — | — | 1 |
| *Candida* sp.3 | 4 | — | 1 | 4 |
| *Candida* sp.4 | 2 | — | — | 2 |
| *Cryptococcus albidus* | 1 | 1 | 1 | 1 |
| *C. flavus* | 2 | — | — | 1 |
| *Debaryomyces hansenii* | 16 | — | — | 13 |
| *Issatchenkia orientalis* | 2 | 1 | — | 1 |
| *Kluyveromyces lactis* | 10 | — | — | 10 |
| *Pichia subpelliculosa* | 1 | — | — | 1 |
| *Rhodotorula glutinis* | 1 | 1 | 1 | — |
| *R. mucilaginosa* | 2 | 1 | 1 | 1 |
| *Torulaspora delbrueckii* | 25 | 3 | — | 2 |
| *Zygosaccharomyces rouxii* | 5 | 1 | — | — |
| Total | 86 | 15 | 4 | 38 |

Welthagen and Viljoen 1999). The occurrence of a limited number of species confirms that the cheese ecosystem is characterized by specific environmental conditions and composition, which promotes selection of a uniform and well-defined microbiota, at least for some technological characteristics (Jakobsen and Narvhus, 1996).

From 176 yeast isolates, 8.5% had the ability to produce protease, 2.3% produced lipase and 21.6% produced ($\beta$-galactosidase. The yeasts *C. albidus* and *R. mucilaginosa* showed noticeable proteolytic, lipolytic and ($\beta$-galactosidase activities. Various strains hydrolyzed lactose, among which were several *Candida* spp., *D. hansenii* and *K. lactis* (Table 2). These results are in accordance with those of other researchers who isolated strains of *R. glutinis, T. delbrueckii, C. catenulata* and *D. hansenii* producing proteases and lipases in milk and milk products (Welthagen and Viljoen 1999; Pereira-Dias et al. 2000). Production of lipolytic and proteolytic enzymes by yeasts in cheese would contribute to the development of the characteristic flavor and smell during the ripening process. Nevertheless, proteolysis and lipolysis of milk and milk constituents could have a significant impact on their quality, promoting a rancid flavor and alterations in product texture (Roostita and Fleet 1996).

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