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The influence of seasons and ripening time on yeast communities of a traditional Brazilian cheese

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ABSTRACT

The occurrence and effects of the dry and rainy seasons on yeast populations in traditional Serro Minas cheese, one of the most popular cheeses produced from raw milk in Brazil, were studied over the course of 60 days of ripening. Enzymatic activity exhibited by these yeast isolates was also studied. A total of 19 yeast species were identified via sequence analysis of the D1/D2 domains of the large subunit of the rRNA gene. Fourteen yeast species were obtained from cheese produced during the dry season, and fifteen species were obtained from cheese produced during the rainy season. High diversity indices for the yeast species were determined for cheese manufactured during both seasons (average $H'_D = 1.7$ and $H'_R = 1.5$, respectively). The predominant species in Serro Minas cheese included *Debaryomyces hansenii*, *Kodamaea ohmeri* and *Kluyveromyces marxianus*. *D. hansenii* 28.12 showed low lipolytic and high proteolytic activity. *K. marxianus* 83F and 60P demonstrated lipolytic and β-galactosidase activity, respectively. *K. ohmeri* 88A displayed low lipolytic and β-galactosidase activity. Maximal lipase, β-galactosidase and protease activity was observed at 20 °C and pH 6.0, 30 °C and pH 7.0 and 50 °C and pH 6.0, respectively. Considering that *D. hansenii* 28.12, *K. ohmeri* 88A and *K. marxianus* 60P together showed protease, lipase and β-galactosidase activity in this study, further research on the possibility of including these yeasts as part of a starter culture and research on their effects on the sensory properties of Serro Minas cheese merit more study.

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1. Introduction

Traditional Minas cheese is an artisanal cheese manufactured by farmers on a small scale, utilizing traditional practices and raw bovine milk. Serro cheese is a variety produced in the central northeast of the state of Minas Gerais, Brazil. This cheese is very popular in Brazil and is considered to be a diversification of semi-hard cheese because it has a typical, markedly acidic taste (Cardoso et al., 2013; Furtado, Mosquim, Fernandes, & da Silva, 2003).

Serro Minas cheese is traditionally made using "pingo" (a natural fermentation starter) and industrial liquid rennet. The natural starter ("pingo") is produced by adding whey from an older cheese that is covered with salt. Species of *Lactobacillus*, *Lactococcus* and *Streptococcus* are found in this natural starter (Borelli, Ferreira, Lacerda, Franco, & Rosa, 2006; Lacerda et al., 2011). The ripening period lasts about three days at room temperature and is followed by refrigeration at approximately 10 °C. Brazilian legislation requires a period of 60 days of maturation for cheese made with raw milk (Brasil, 2000). However, most producers do not follow this requirement and sell the cheese after a shorter ripening period (between 3 and 15 days).

Cheese ripening is a complex phenomenon involving a wide range of biochemical reactions. High microbial counts are present in the cheese throughout the ripening process, and the composition of the microbial population plays a significant role in the maturation process (Beresford, Fitzsimons, Brennan, & Cogan, 2001). The important contribution made by secondary microbiota (mainly constituted by enterococci, micrococci, non-starter lactic acid bacteria and yeasts) to cheese maturation is well recognized (Beresford et al., 2001). In particular, yeasts are associated with the secondary microbiota of a wide variety of cheeses and can play an important role in the ripening of such cheeses. Investigations of the yeast composition in cheese reveals a large diversity of species belonging to the *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Kodamaea*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Torulaspora*, *Trichosporon*, *Yarrowia* and *Zygosaccharomyces* genera. Although the prevalence of different yeast species depends on the type of the cheese under consideration, *Debaryomyces hansenii* is one

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of the most common species isolated (Büchl & Seiler, 2011). The occurrence of this species and other yeasts with high counts in cheese is attributable to their tolerance of low pH, reduced water activity and high salt concentrations, as well as to their ability to grow at the low storage temperatures that characterize the ripening environment (Ferreira & Viljoen, 2003). In addition, they are widely dispersed in the dairy environment and appear as natural contaminants in raw milk, air, dairy implements, surfaces, equipment, brine and smear water (Mounier et al., 2006).

Yeasts are involved both directly and indirectly in the cheese ripening process (Fleet, 2011; Jakobsen & Narvhus, 1996). The consumption of lactate, formation of alkaline metabolites, fermentation of lactose, lipolysis, proteolysis and the formation of aromatic compounds are some of the yeast activity that are important for the typical characteristics of some cheese varieties (Gardini et al., 2006; Rossi, Gobbetti, Smacchi, Battistotti, & Fox, 1998). For the reasons explained above and because of their positive interactions with lactic acid bacteria starters, yeasts are also considered to be potential cheese adjunct cultures (De Freitas, Pinon, Maubois, Lortal, & Thierry, 2009). In contrast, yeasts may cause product spoilage such as the production of gas, off-flavors, softening, discoloration and swollen packages in semi-soft and soft cheeses (Fleet, 2011).

Changes in the total yeast population over the maturation period have been monitored for several cheese varieties (Beresford & Williams, 2004), but few results exist regarding the manner in which the yeast diversity may change because of seasonal variations. There is evidence that the yeast populations of the traditional Greek Anevato, Camembert and Brie cheeses were affected by the season during which they were manufactured (Hatzikamari, Litopoulou-Tzanetaki, & Tzanetakis, 1999; Viljoen, Khoury, & Hattingh, 2003). Although there is considerable information on the size and species composition of the yeast populations of many cheeses, there is very little information on the changes in species throughout the ripening process. The present study was conducted using the traditional Brazilian Serro Minas cheese manufactured during the dry and rainy seasons and ripened over 60 days to determine the frequency and seasonal diversity of the yeasts during the ripening of the cheese and to characterize the yeasts with regard to several technological properties of some useful isolated strains.

2 . Materials and methods

2.1 . Sample collection

A total of 100 samples of Serro cheese were obtained from five farms; 50 samples were collected during the dry season (winter in Brazil) and 50 samples were collected during the rainy season (summer in Brazil). The samples collected during the dry season were from cheeses manufactured in May and June 2008, and the samples collected during the rainy season were from cheeses manufactured in January 2009. Ten cheese samples were collected from each farm during each season after two days of ripening; these cheeses were then stored without packaging in a refrigerated room (approximately 10 °C) of a storage facility throughout the 60-day ripening period. The cheeses were aseptically turned every day. The average temperature and relative humidity (RH) of the refrigerated room during the ripening periods were 10.5 °C and 88.2% RH during the dry season and 11.5 °C with 81.3% RH, respectively, during the rainy season. Two cheeses from each farm and season, stored in the refrigerated room, were sampled after 3, 15, 30, 45 and 60 days of ripening. The samples were then transported to the laboratory under refrigeration for microbiological analyses.

2.2 . Enumeration and isolation of yeast strains

The samples (25 g each) were homogenized with 225 mL of 0.1% peptone water in a Stomacher 400 lab blender (Seward, London, UK) for 1 min, and serial decimal dilutions were prepared using the same diluent. Aliquots (100 μL, in triplicate) of appropriate decimal dilutions were spread on yeast extract-malt extract agar plates (YMA — glucose, 10 gL^{-1}; peptone, 5 gL^{-1}; yeast extract, 3 gL^{-1}; malt extract, 3 gL^{-1}; agar, 20 gL^{-1}) containing 200 mg L^{-1} of chloramphenicol and incubated at 25 °C for 5 days. After growth was noted, the yeast colonies of each different morphotype were counted. The yeasts that displayed different morphologies were chosen for isolation and later identification. When possible, at least three randomly selected colonies of each different morphotype were purified by repeated streaking on YMA plates and preserved at −80 °C in GYMP broth (glucose, 20 gL^{-1}; yeast extract, 5 gL^{-1}; malt extract, 10 gL^{-1}; and NaH_2PO_4, 2.0 gL^{-1}) with 20% (v/v) glycerol or with liquid nitrogen, for further identification.

2.3. Yeast identification

The yeasts were preliminarily grouped according to their colony morphology and physiological tests commonly used for identification, performed according to the procedures described by Kurtzman, Fell, Boekhout, and Robert (2011), including: fermentation of carbohydrates, growth on various carbon and nitrogen sources, growth on media with a high concentration of sugar and sodium chloride, growth at various temperatures, tolerance to 1% acetic acid, formation of extracellular amyloid compounds and resistance to 0.01% cycloheximide. Physiology-based groupings were confirmed via PCR fingerprinting using the synthetic oligonucleotide $(GTG)_5$ as the microsatellite DNA sequence (Ali, Muller, & Epplen, 1986). DNA extraction was achieved according to the modified procedure of Brandão et al. (2011). Yeast colonies were grown on YMA at 25 °C overnight, transferred to 2-mL sterile Eppendorf tubes containing 100 μL of extraction buffer solution (50 mmol Tris–HCl L^{-1}, 100 mmol NaCl L^{-1}, 5 mmol EDTA L^{-1}, and 1% w/v SDS, at pH 8) and incubated at 65 °C for 30 min. After incubation, 100 μL of phenol/chloroform/isoamilic alcohol (25:24:1, Sigma) solution was added. The mixture was mixed vigorously with a vortex stirrer and centrifuged for 15 min at 14,000 rpm. The supernatant was withdrawn, transferred to another 2-mL sterile Eppendorf tube, and 100 μL of cool ethanol (70% v/v) was added. The mixture was also vortexed vigorously, and centrifuged for 15 min at 14,000 rpm. The DNA was dried overnight at room temperature, suspended in 50 μL TE-buffer (10 mmol Tris–HCL and 1 mmol EDTA) and stored at −20 °C until processing. The PCR reactions were performed as described by Libkind et al. (2003). Yeast strains with identical DNA band patterns were grouped and putatively considered to belong to the same species (Gadanho & Sampaio, 2002). The sequence of the D1/D2 domains of the large subunit of the rRNA gene of at least one representative strain of each PCR fingerprinting group was determined.

The D1/D2 variable domains of the large subunit of the rRNA gene were amplified as described previously by Lachance, Bowles, Starmer, and Barker (1999) using the primers NL-1 (5′-GCATATCAATAAGCGG AGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′). Sequencing of the D1/D2 domains was performed directly from purified PCR products using a MegaBace™ 1000 automated sequencing system (Amersham Biosciences, USA). The sequences were compared with other rDNA sequences deposited in the GenBank using the NCBI BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST). According to Kurtzman et al. (2011), conspecific strains differ by no more than three nucleotides among the 500–600 nucleotides of the D1/D2 domains. Differences of six or more nucleotides (1%) indicate that the strains represent different species.

2.4. Preliminary plate screening of the protease, lipase and β-galactosidase-producing yeast strains

The amount of extracellular protease production was determined according to the method described by Buzzini and Martini (2002), with modifications of the pH of the YPD agar (glucose, 20 gL^{-1}; yeast extract, 10 gL^{-1}; peptone, 10 gL^{-1}; and agar, 20 gL^{-1}) and the

incubation temperature. Calibrated suspensions ($A_{580} = 0.5$, corresponding to an average cell concentration of 10^6 cells mL^{-1}) of 24-h yeast cells were used to inoculate the solidified agar surface of YPD containing 20 gL^{-1} of casein solution, at pH 5.5 (average pH of Serro Minas cheese). The plates were incubated at 10 °C (ripening temperature of Serro Minas cheese) for 2–5 days. A clear zone around the colony indicated protease activity. To determine the lipase activity, a pre-culture of the yeasts in YPD was initially grown at 25 °C for 48 h. Purified colonies were punctually inoculated on lipase agar according to the method described by Colen, Junqueira, and Moraes-Santos (2006), with modifications in the composition of the medium and the incubation temperature. The lipase agar contained meat peptone, 25 gL^{-1}; casein peptone, 25 gL^{-1}; yeast extract, 1.0 gL^{-1}; $NaNO_3$, 1.0 gL^{-1}; $MgSO4 \cdot 7H_2O$, 5.0 gL^{-1}; KH_2PO_4, 1.0 gL^{-1}; and 5 mL L^{-1} of olive oil at pH 6.0. The plates were incubated at 10 °C for 3 to 5 days. A clear zone around the colony indicated lipolytic activity. The yeast strains were tested for the production of β-galactosidase on Yeast Nitrogen Base (Difco, USA) with 5 gL^{-1} of lactose as the sole carbon source (Kurtzman et al., 2011). Positive growth was considered as the ability to produce the enzyme.

2.5. Cultivations in olive oil, casein and lactose and determination of specific growth rates

Isolates belonging to each of the three yeast species most prevalent on Serro Minas cheese were inoculated to an initial $OD_{600\ nm}$ of 0.2 in specific media for lipase, protease and β-galactosidase production (shown in the next sections). The specific media (initial pH of 6.0) were incubated at 20 °C and 160 rpm for 72 h. Yeast growth was followed spectrophotometrically at 600 nm and the specific growth rate was determined during the exponential growth phase. The cultivations were carried out in duplicate for each microorganism.

2.6. Production and characterization of lipase, protease and β-galactosidase enzymes

The selected yeast isolates were pre-grown in YPD liquid medium (initial pH 6.0) at 30 °C and 150 rpm for 24 h. After the cells were harvested by centrifugation (14,000 rpm for 15 min) and inoculated to an initial $OD_{600\ nm}$ of 0.2 in specific media for lipase, protease and β-galactosidase production, the cultivations were carried out in duplicate for each microorganism.

2.6.1. Production of lipase in liquid medium and lipolytic activity assay

For lipase production, the following liquid growth medium was used according to the procedure described by Colen et al. (2006), with modifications in the composition of the medium: meat peptone, 25 gL^{-1}; yeast extract, 1.0 gL^{-1}; $NaNO_3$, 1.0 gL^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 gL^{-1}; KH_2PO_4, 1.0 gL^{-1}; 5 mL L^{-1} of olive oil, and pH 6.0 (adjusted with citrate phosphate buffer 0.1 mmol L^{-1}), at 20 °C and 160 rpm for 72 h. The cells that grew were harvested by centrifugation, and the cell-free supernatants were used to determine the corresponding extracellular lipolytic activity.

The spectrophotometric lipase assay was performed using *p*-nitrophenyl palmitate (pNPP) according to the method described by Winkler and Stuckmann (1979), with modifications. The substrate solution was prepared by adding 1.0 mL of solution A (30 mg of pNPP in 10 mL of propane-2-ol) to 9 mL of solution B (0.1 g of gum arabic and 0.4 g of Triton X-100 in 90 mL of buffer) drop wise, with intense stirring. The assay mixture consisted of 160 μL of substrate solution and 40 μL of suitably diluted enzyme. The assay mixture was incubated at various temperatures for 15 min and the p-nitrophenol released was measured at 410 nm in the spectrophotometer. The optimal pH of the enzyme was determined by measuring the lipase activity at 20 °C at various pH levels (pH 4–10), and citrate phosphate buffer (0.1 mmol L^{-1}, pH 3.0–6.0) and Tris–HCl buffer (0.1 mmol L^{-1}, pH 8.0–10.0) were added. The optimal temperature of the enzyme was determined by measuring the enzyme activity at various temperatures (20–60 °C) in 0.1 mmol L^{-1} of citrate phosphate buffer, at pH 6.0. The absorbance was measured at 410 nm against a control. All assays were performed in triplicate. One enzyme unit is defined as the quantity necessary to release 1 μmol of *p*-nitrophenol from the substrate per milliliter per minute. Under the conditions described, the extinction coefficient of *p*-nitrophenol is $E_{410} = 1.275 \times 10^4$ $mol \cdot L^{-1}$ cm^{-1}.

2.6.2. Production of protease in liquid medium and proteolytic activity assay

For protease production, the following liquid growth medium was used: KH_2PO_4, 1.5 gL^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 gL^{-1}; KCl, 0.5 gL^{-1}; $FeSO_4$, 0.001 gL^{-1}; and 1.0 gL^{-1} of casein solution, according to the method described by Pontecorvo, Roper, Hemons, Macdonald, and Bufton (1953), at pH 6.0 (adjusted with citrate phosphate buffer 0.1 mmol L^{-1}), 20 °C and 160 rpm for 72 h. The cells that grew were harvested by centrifugation, and the cell-free supernatants were used to determine the corresponding extracellular proteolytic activity.

Proteolytic activity was determined according to the method described by Charney and Tomarelli (1947). Appropriately diluted enzyme extract (100 μL) was added to 100 μL of 25 gL^{-1} Azocasein solution. The optimal pH of the enzyme was determined by measuring the protease activity at 40 °C at various pH levels (5.0–10.0) by adding citrate phosphate buffer (0.1 mmol L^{-1}, pH 5.0–7.0) and Tris–HCl buffer (0.1 mmol L^{-1}, pH 8.0–10.0). The optimal temperature of the enzyme was determined by measuring the enzymatic activity at various temperatures (20–60 °C) in 0.1 mmol L^{-1} citrate phosphate buffer, at pH 6.0. The reaction was stopped with the addition of 800 μL of 50 gL^{-1} trichloroacetic acid (TCA), and the remaining residue was removed by centrifugation (14,000 rpm for 5 min). A total of 500 μL of 0.5 mol L^{-1} KOH solution was added to the supernatant. The absorbance was measured at 445 nm against an enzyme-free control. One enzyme unit is defined as the amount of enzyme that promotes a difference of 0.01 absorbance units per minute between the enzyme sample and the control, under the assay conditions.

2.6.3. Production of β-galactosidase in liquid medium and β-galactosidase activity assay

To select the best β-galactosidase-producing yeast, the yeast cells were cultivated in a 250-mL shake flask with 50 mL of medium containing yeast extract, 33.2 gL^{-1}; K_2HPO_4, 66.4 gL^{-1}; $NH_4H_2PO_4$, 33.2 gL^{-1}; $(NH_4)_2HPO_4$, 33.2 gL^{-1}; $MgSO_4 \cdot 7H_2O$, 33.2 gL^{-1}; and 25 gL^{-1} of lactose, according to the method described by Mahoney, Nickerson, and Whitaker (1975), at pH 6.0 (adjusted with citrate phosphate buffer 0.1 mmol L^{-1}), 20 °C and 160 rpm for 72 h. The cells were permeabilized prior to adding them to the assay of intracellular β-galactosidase. A volume of resuspended cells in 0.2 mmol L^{-1} phosphate buffer (pH 6.0) amounting to 10–20 mg of dry cell mass was mixed with 5 mL isoamyl alcohol and diluted up to 25 mL with 0.2 mmol L^{-1} phosphate buffer at pH 6.0. The mixture was shaken for 15 min at room temperature to make the cell envelopes permeable and was used for the enzyme assay (Barberis & Gentina, 1998). For determination of β-galactosidase activity, the method described by the Food Chemical Codex (1993) was used, with some modifications. The substrate for the enzymes was 2.5 mg mL^{-1} of *o*-nitrophenol-β-D-galactopyranoside (ONPG, Sigma, N-1127), which was prepared in 0.1 M phosphate buffer. One milliliter of the crude permeabilized cell suspension with isoamyl alcohol was incubated with 4 mL of the above substrate mixture for 15 min.

The optimal pH of the enzymes was determined by measuring the β-galactosidase at 30 °C at various pH levels (pH 5–10) in citrate phosphate buffer (0.1 mmol L^{-1}, pH 5.0–7.0) and Tris–HCl buffer (0.1 mmol L^{-1}, pH 8.0–10.0). The optimal temperature of the enzymes was determined by measuring the enzyme activity at various temperatures (20–60 °C) in 0.1 mmol L^{-1} of citrate phosphate buffer, at pH 7.0.

The reaction was stopped by adding 1 mL of 1 mmol L^{-1} Na_2CO_3, and the absorbance was measured at 420 nm. The ONP concentration was calculated using an extinction coefficient of 4.2371 mL μmol^{-1} cm^{-1}. One unit (U) of enzyme activity was defined as the enzyme quantity that liberated 1 μmol of *o*-nitrophenol per minute under the assay conditions.

2.7. Statistical analyses

Two-way analysis of variance (ANOVA) was performed. Tukey's multiple comparisons of means test and the Bonferroni test were used to identify differences between the means. The statistical analysis was performed using the R Project for Statistical Computing, version 2.15.1 software. The level of statistical significance was set at $p < 0.05$.

2.8. Yeast community diversity

Classical ecology indexes were used to determine the species richness (S). The Shannon–Wiener index (H′) indicates general biodiversity and the Simpson's index (D) indicates dominance of the species in each ripening period as follows:

$$S = \text{number of species};\ H' = -\sum p_i \ln p_i;\ D = \sum n(n-1)/N(N-1)$$

where S is the number of species and p_i is the proportion of isolates of the species i, estimated as n_i/N, where n_i is the total number of organisms of a particular species, and N is the total number of organisms of all species. Further information about these indexes can be found in Magurran (2011). Cluster analysis of the 61 different molecular patterns obtained via PCR fingerprinting with the primer $(GTG)_5$ was generated using an unweighted pair group algorithm with arithmetic averages (UPGMA). Clustering was carried out using the computer program PAST, version 1.90 (Hammer, Harper, & Ryan, 2001).

3 . Results and discussion

3.1. Yeast enumeration and identification during the ripening of cheese

A total of 243 yeast isolates were obtained during the ripening of the Serro Minas cheese. At least 2–3 isolates representing different morphotypes were selected per sample. Physiological tests were performed on these 243 yeast isolates to group them according to their physiological profiles. After obtaining 202 different physiological profiles, these yeasts were grouped using PCR fingerprinting with the primer $(GTG)_5$ in 61 different molecular patterns (see S-Fig. 1 in the supplement file) that were selected for sequence analysis of the D1/D2 domains of the 26S rRNA gene. Nineteen yeast species were identified. The occurrence of yeasts during ripening in Serro Minas cheese manufactured during the dry and rainy seasons is shown in Table 1. Three replicates of yeast counts for each sample were used for analysis of variance (ANOVA). The results of this analysis showed no interaction between the seasons and ripening time factors with respect to the yeast counts ($p = 0.6369$). No significant difference between dry and rainy seasons ($p = 0.9564$) was observed in the mean of the total yeast counts for each ripening time. Similarly, Psoni, Tzanetakis, and Litopoulou-Tzanetaki (2003) did not find differences between seasons in the yeast counts of Batzos cheese, a traditional Greek cheese. Caridi, Micari, Foti, Ramondino, and Sarullo (2003) observed a higher yeast count in the artisanal Caprino d'Aspromonte cheese manufactured during the winter than in that manufactured during summer. Viljoen et al. (2003) described a difference in the population of yeasts of Camembert and Brie cheeses produced during the winter of one to two orders of magnitude higher than that observed in these cheeses produced during the summer. According to Tamagnini, de Sousa, González, and Budde (2006), yeasts in Crottin cheese, which is produced in Argentina, also

Table 1
Average numbers of yeast species populations ($\log_{10}$ cfu·g^{-1}) isolated during the cheese ripening periods and seasons (dry and rainy) in traditional Serro Minas cheese produced in Brazil.

	Days of ripening				
Yeast species	3*	15*	30*	45*	60*
Dry season					
Candida atlantica					6.1 (1)[A]
C. catenulata		6.0 (1)	7.1 (3)		5.5 (1)
C. intermedia	4.4 (1)			6.4 (2)	
C. phangngensis		7.3 (1)		4.5 (1)	
C. silvae	5.7 (1)				
Debaryomyces hansenii	5.6 (2)	7.2 (7)	8.1 (7)	6.6 (2)	7.8 (12)
Galactomyces geotrichum				5.0 (2)	
Kluyveromyces lactis			7.0 (2)	7.0 (3)	6.2 (2)
K. marxianus	4.5 (1)	6.0 (2)	6.9 (4)	7.1 (5)	6.0 (3)
Kodamaea ohmeri	4.2 (6)	6.2 (6)	7.4 (6)	5.6 (2)	6.6 (2)
Saccharomyces cerevisiae	4.1 (1)				
Torulaspora delbrueckii	6.2 (2)	6.8 (1)	6.6 (2)		5.5 (1)
Trichosporon sp.	3.5 (1)	4.8 (1)			
T. montevideense			5.5 (1)		
Mean[B]	5.51[a]	6.82[b]	7.42[b]	6.65[a, b]	7.00[b]
Rainy season					
Candida sp.	3.5 (1)				
C. catenulata	3.5 (1)	5.8 (1)			6.6 (4)
C. parapsilosis	2.5 (1)			6.4 (2)	
C. tropicalis	4.5 (1)				
Debaryomyces hansenii	4.6 (3)	8.1 (7)	8.3 (5)	7.8 (8)	7.6 (6)
Galactomyces candidum				5.1 (1)	
G. geotrichum				5.1 (1)	
Kluyveromyces lactis			5.5 (1)		6.8 (2)
K. marxianus	6.0(3)		6.3 (3)	6.9 (4)	7.1 (3)
Kodamaea ohmeri	5.7 (18)	6.9 (5)	6.4 (1)	6.5 (7)	5.9 (5)
Rhodotorula mucilaginosa		5.5 (1)			
Saccharomyces cerevisiae	4.5 (1)				
Torulaspora delbrueckii	5.5 (1)	6.7 (1)	6.9 (3)	4.8 (1)	
Trichosporon sp.		6.5 (1)			
T. montevideense	2.8 (1)				
Mean[B]	5.28[a]	7.42[b]	7.61[b]	7.02[b]	7.11[b]

*number of samples/ripening time ($n = 10$).
[A]Number in parenthesis represents the number of isolates of each yeast species.
[B]Mean of total yeast species/ripening time.
[a, b]Within a row, different superscript lowercase letters denote significant differences ($p < 0.05$) among the various periods of storage for each season studied (Tukey's test).

showed distinct patterns while being stored during different seasons. After ripening for 15 to 36 days, the yeast counts in Crottin cheese gradually increased during the fall, decreased during the summer and did not change during the winter. In the present study, there was a significant difference in the mean of the total yeast counts between the days on which the samples were obtained in each season studied ($p < 0.0001$). Significant differences in the yeast counts occurred at 15 days of ripening. The mean number of total yeast populations in the cheese produced during the dry season increased significantly, ranging from 5.51 ± 4.84 log cfu·g^{-1} to 6.82 ± 6.07 log cfu·g^{-1} after 3 to 15 days of ripening, then remained without significant differences until the end of the ripening period of 60 days (Table 1). During the rainy season, the mean number of total yeast populations increased significantly from 5.28 ± 4.52 log cfu·g^{-1} to 7.42 ± 6.97 log cfu·g^{-1} after ripening for 3 to 15 days, and also remained stable until the end of ripening. Studies on the evolution of yeast populations in the cheese indicated that the highest number of yeasts observed after seven days of ripening was 8–9 log cfu·g^{-1} (Eliskases-Lechner & Ginzinger, 1995). Viljoen et al. (2003) also described an increase in the number of yeasts on the surface of Camembert and Brie cheeses from 6 log cfu·g^{-1} after seven days of ripening to populations as high as 8 log cfu·g^{-1} after ripening for 15 days during the winter.

Ninety-eight yeast isolates obtained from Serro Minas cheese produced during the dry season were identified as belonging to 14 species. During the rainy season, a total of 104 yeast isolates, belonging to 15 species, were obtained (Table 1). *Candida atlantica*, *Candida intermedia*,

Candida phangngensis and *Candida silvae* were isolated in only cheese manufactured during the dry season, whereas *Candida* spp., *Candida parapsilosis*, *Candida tropicalis*, *Galactomyces candidum* and *Rhodotorula mucilaginosa* were isolated in only cheese produced during the rainy season.

The most prevalent yeast species isolated from Serro Minas cheese were *D. hansenii*, *Kodamaea ohmeri* and *Kluyveromyces marxianus*. *Debaryomyces hansenii* and *K. ohmeri* were the prevalent species in Serro Minas cheese manufactured during both seasons in all the ripening periods studied. *Debaryomyces hansenii* counts increased almost four orders of magnitude from 3 to 15 days of ripening during the rainy season, reaching 8.1 log $cfu \cdot g^{-1}$ after 15 days (Table 1). High populations of *D. hansenii* may develop in soft, semi-soft, and even Cheddar-style cheese during the maturation stage and are considered to have positive influences on cheese quality (Fleet, 2011). Moreover, recent studies have indicated that *D. hansenii* could successfully be used as part of starter cultures for cheese manufacturing, in which it can enhance flavor development during cheese maturation (Sørensen et al., 2011). *Kodamaea ohmeri* was the most prevalent species during the rainy season and was isolated throughout the ripening period. This result suggests that this species may play a fundamental role in the ripening of Serro Minas cheese. Borelli et al. (2006) showed that this species was the fourth most prevalent species in artisanal Canastra Minas cheese produced in Minas Gerais, Brazil. The presence of *K. ohmeri* in two different artisanal cheeses produced in the state of Minas Gerais (Serro and Canastra Minas cheeses) indicates that this species is active in the yeast community responsible for the production of these traditional cheeses. This species is frequently isolated in fruit and vegetable fermentation industries (de Miguel-Martinez et al., 2013) and was also isolated in the natural starter ("pingo") used in the manufacture of Canastra Minas cheese. Additionally, according to de Miguel-Martinez et al.(2013), this yeast species is considered to be an opportunistic pathogen.

During the dry season, *D. hansenii*, *K. ohmeri* and *K. marxianus* were detected in cheeses from all the periods of storage studied (Table 1). *Kluyveromyces marxianus* was the only species not found on cheeses after 15 days of ripening during the rainy season. According to Fleet (2011), high populations of *K. marxianus* in cheese during the maturation stage are considered to have positive influences on cheese quality. *K. marxianus* is one of the dominant species in the yeast community of Camembert cheeses, and its principal activity is the consumption of lactose (Baudrit, Sicard, Wuillemin, & Perrot, 2010). Species of *Kluyveromyces* produce β-galactosidase that enables them to utilize lactose, which is an attribute of particular importance for the dairy industry (Johnson & Echavarri-Erasun, 2011).

Torulaspora delbrueckii was present during almost all the ripening period except at 45 days (during the dry season) and at 60 days (during the rainy season). *T. delbrueckii* was also isolated by Welthagen and Viljoen (1998) in Gouda cheese after 32 days of ripening and by Westall and Filtenborg (1998) as a major part of the yeast community in Danish Feta cheese. Fadda, Mossa, Pisano, Delphano, and Cosentino (2004) found this yeast on Fiore Sardo cheese after 48 h and one month of ripening. Borelli et al. (2006) isolated this species in natural starter, cheese curd and in traditional Canastra Minas cheese ripened for five days. Golié et al. (2013) found *T. delbrueckii* as one of the predominant yeast species in white pickled artisanal cheeses collected in Serbia.

Candida catenulata was detected during three ripening periods (15, 30 and 60 days) in cheese manufactured during the dry season and at 3, 15 and 60 days of ripening in cheese manufactured during the rainy season. Borelli et al. (2006) found *C. catenulata* to be one of the most prevalent yeast species in artisanal Canastra Minas cheese. In a study of industrial and artisanal Italian cheeses, Pirisi, Comunian, Urgeghe, and Scintu (2011) detected *C. catenulata* in only artisanal cheeses. According to Sørensen et al. (2011), *C. catenulata* is important in the smear of surface-ripened cheeses produced in Denmark. The other *Candida* species were only sporadically found during the ripening of Serro Minas cheese.

Kluyveromyces lactis was detected only after 30 days of ripening, suggesting that this species may be important in the later stages of maturation. *Trichosporon* sp., *Trichosporon montevideense*, *Saccharomyces cerevisiae*, *G. candidum*, *Galactomyces geotrichum* and *R. mucilaginosa* were seldom detected during the periods of collection and their infrequent discovery suggests that they were most likely transient contaminants of Serro Minas cheese.

3.2. The yeast diversity during the ripening of cheese

Species richness (S), the Shannon–Wiener index (H′) (to estimate the diversity of the yeast species) and the Simpson's index (D), for evaluating dominance of the species, were calculated and are presented in Fig. 1. Species richness was highest in cheeses ripened for three days during the rainy season ($S_R = 10$). These cheeses possessed a higher number of isolated yeasts than was evident during the other periods. The lowest species richness was that of cheeses with 30 and 60 days of ripening during the rainy season, where five species were isolated ($S_R = 5$). Of nineteen yeast species identified in Serro Minas cheese, six species were represented by a single isolate, and 13 species were isolated more than once. The highest general diversity of yeasts (H′) was observed in cheese ripened for 45 days during the dry season ($H'_D = 1.84$), where seven different yeast species were identified and no dominance of any of species was scored ($D_D = 0.12$). The mean values for yeast diversity (H′) found in Serro Minas cheese (average $H' = 1.6$) were higher than those found by Golié et al. (2013),who determined the diversity of yeast species in artisanal cheeses collected in Serbia and Croatia (average $H' = 0.8$).

The composition of yeast species differed during the ripening times of the cheese. *Kodamaea ohmeri* was the principal isolate (40% in cheeses manufactured during the dry season and 58.1% in cheeses manufactured during the rainy season) in cheeses after three days of ripening. *Debaryomyces hansenii* was predominant in cheeses ripened for 15 to 60 days and manufactured during both seasons (28% to 54.5% during the dry season and 30% to 43.8% during the rainy season), with the exception of cheeses manufactured during the dry season and ripened for 45 days, in which *K. marxianus* was predominant (29%).

PCR fingerprinting amplification of the yeast isolates generated different electrophoretic patterns (see S-Fig. 1 in the supplement file). The yeast isolates were grouped into five major clusters (A to E) with a similarity level of approximately 24% (see S-Fig. 2 in the supplement file). The molecular profile of the red yeast *R. mucilaginosa* is not shown in this figure because the species isolate was no longer viable after thawing. The isolates of *D. hansenii* showed a remarkable molecular diversity of their PCR fingerprinting profiles. This result suggests that more than one strain of *D. hansenii* may be involved in the ripening process of Serro Minas cheese.

3.3. Screening for proteolytic, lipolytic and β-galactosidase activity in yeast isolates

Of the 199 yeasts isolated during the ripening of Serro Minas cheese tested for lipase, protease and β-galactosidase enzymes, 5% produced lipase on olive oil-agar medium, 8% produced protease on casein-agar medium, and 54% produced β-galactosidase on lactose-agar medium (Table 2). *Kluyveromyces lactis*, *Trichosporon* spp. and *T. montevideense* displayed noticeable β-galactosidase activity. *Trichosporon* spp. *and T. montevideense* also showed protease and lipase activity. Fifty-four percent of the isolates of *K. marxianus* demonstrated β-galactosidase activity, 7% showed lipolytic activity and only 4% were positive for protease production. *Debaryomyces hansenii*, which was the predominant species in Serro Minas cheese, showed noticeable β-galactosidase activity (97% of the isolates produced this enzyme); almost 10% of the isolates displayed proteolytic activity and nearly 6% produced lipase. According to van den Tempel and Jakobsen (2000), *D. hansenii* originating from Danablu cheese demonstrated strong growth and assimilation of lactose

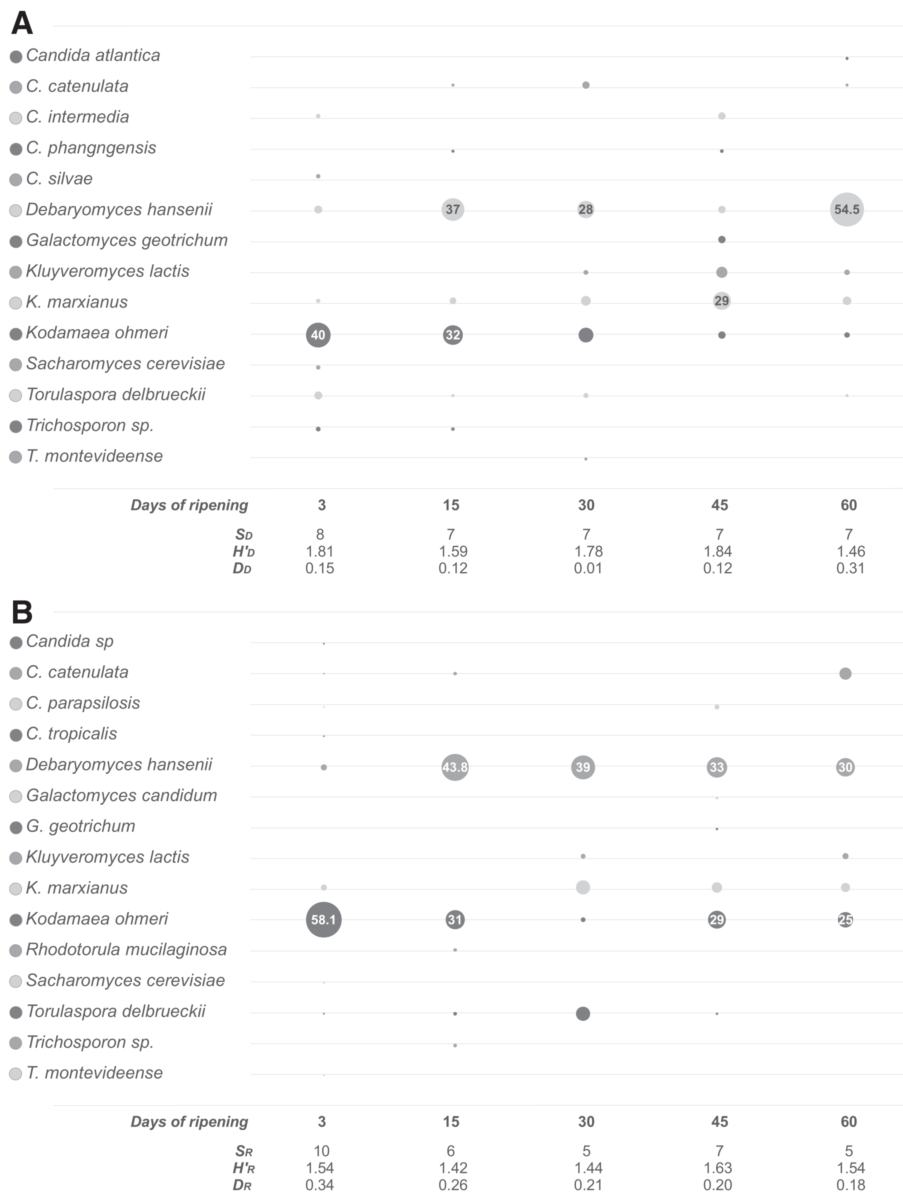

Fig. 1. Relative abundance (%), diversity indices of species richness (S), Shannon–Wiener index (H′) indicating general biodiversity and Simpson's index (D) for evaluating dominance of yeast species in ten cheese samples/ripening time for cheeses manufactured during the dry season (A) and rainy season (B).

and galactose during maturation at 10 °C. According to Fleet (2011), *D. hansenii* also has the capacity to produce proteases and extracellular lipases. It is well recognized that *D. hansenii* metabolizes lactose, utilizes multiple carbon and nitrogen sources, and generates an alcoholic, acidic and cheesy flavor (Golić et al., 2013; Masoud & Jakobsen, 2002, 2005; Welthagen & Viljoen, 1999). *Kodamaea ohmeri* also demonstrated β-galactosidase activity (26% of the isolates tested), proteolytic activity (12% of the isolates) and lipolytic activity (3% of the isolates). *Torulaspora delbrueckii* showed only β-galactosidase activity (27% of the isolates). These results are similar to those found by Welthagen and Viljoen (1998, 1999), who reported that *T. delbrueckii* isolated during the manufacture of Cheddar and Gouda cheeses did not present proteolytic or lipolytic activity. According to Borelli et al. (2006), 8% of the *T. delbrueckii* strains isolated during the production of traditional Canastra cheese produced β-galactosidase. Some *Candida* species (*C. catenulata*, *C. intermedia* and *C. parapsilosis*) demonstrated β-galactosidase activity. All the *C. intermedia* isolates were positive for β-galactosidase activity. *Candida* spp. exhibited proteolytic and lipolytic activity. *Rhodotorula mucilaginosa* showed only proteolytic activity. All the *G. candidum* isolates showed proteolytic and lipolytic activity, and strains of *G. geotrichum* produced lipase. *Geotrichum candidum* strains isolated from Armada cheese can usually be considered highly lipolytic (Sacristán, González, Castro, Fresno, & Tornadijo, 2012). According to Chebeňová-Turcovská, Ženišová, Kuchta, Pangallo, and Brežná (2011), *G. geotrichum* is known to participate in proteolysis and flavor formation of Slovakian bryndza cheese. Our results demonstrated the β-galactosidase activity of the yeasts found in Serro Minas cheese, as well as their proteolytic and lipolytic potential.

3.4. Selection and quantitative characterization of proteolytic, lipolytic and β-galactosidase yeast

An isolate of the three yeast species that were predominant in Serro Minas cheese that demonstrated lipase, protease and/or β-galactosidase activity on solid medium were tested for their ability to produce these enzymes in a specific liquid medium (pH 6.0) containing olive oil, lactose or casein, at 20 °C for 72 h. In previous experiments performed for isolates of *D. hansenii*, *K. marxianus* and *K. ohmeri* in liquid medium

Table 2
Production of protease, lipase and β-galactosidase on solid medium by the yeasts isolated from the traditional Brazilian Serro Minas cheese.

Species	Number of isolates tested	Number of enzyme-producing isolates		
		Protease	Lipase	β-galactosidase
Candida sp.	1	1	1	–
Candida atlantica	1	–	–	–
C. catenulata	10	–	1	4
C. intermedia	2	1	–	2
C. parapsilosis	3	–	2	1
C. phangngensis	2	–	1	–
C. silvae	1	–	–	–
C. tropicalis	1	–	–	–
Debaryomyces hansenii	62	6	4	60
Galactomyces candidum	1	1	1	0
G. geotrichum	2	–	1	–
Kluyveromyces lactis	9	–	–	9
K. marxianus	28	1	2	15
Kodamaea ohmeri	57	7	2	15
Rhodotorula mucilaginosa	1	1	–	–
Saccharomyces cerevisiae	2	–	–	–
Torulaspora delbrueckii	11	–	–	3
Trichosporon sp.	3	1	1	3
T. montevideense	2	2	1	2
Total	199	16	11	107

containing olive oil and casein at pH 5.0 and 6.0, and at 10 °C and 20 °C, protease and lipase activity were higher at pH 6.0 and 20 °C(data not shown). *D. hansenii* 28.12, *K. marxianus* 83 F and *K. ohmeri* 88A were characterized for their ability to grow in olive oil medium (Fig. 2A) and the specific growth rates of these three yeasts were determined in this medium (Table 3). *K. marxianus* 83F revealed the highest specific growth rate in olive oil (0.13 h^{-1}), followed by *K. ohmeri* 88A. In contrast, *D. hansenii* 28.12 grew slower in olive oil but reached approximately the same cell density as the other yeasts tested after 72 h (Fig. 2A).

K. marxianus 60P, *D. hansenii* 28.12 and *K. ohmeri* 88A were characterized for their ability to grow in a lactose medium (Fig. 2B). Although *D. hansenii* 28.12 demonstrated the highest specific growth rate in lactose (0.14 h^{-1}), it achieved a low cell density after 72 h, whereas *K. marxianus* 60P and *K. ohmeri* 88A showed a high cell density after 72 h. The three yeast species achieved high specific growth rates (Table 3), suggesting that all species assimilate lactose. One defining feature of *K. marxianus* is their capacity to utilize lactose as a carbon source, as mentioned previously.

D. hansenii 28.12, *K. marxianus* 93K and *K. ohmeri* 33H were characterized for their ability to grow in a casein medium (Fig. 2C). *D. hansenii* 28.12 achieved the highest specific growth rate in casein (0.10 h^{-1}) and reached the highest cell density after 72 h in comparison with *K. marxianus* 93K and *K. ohmeri* 33H, which grew little in casein.

Lipase activity ($U \cdot mL^{-1}$) was determined after growth of *D. hansenii* 28.12, *K. marxianus* 83F and *K. ohmeri* 88A in olive oil (pH 6.0) at 20 °C for 72 h. The effect of temperature on lipase activity was demonstrated at temperatures ranging from 20 °C to 60 °C (pH 6.0) and the optimum temperature of the lipase produced by the three yeast species was 20 °C (Fig. 3A). The optimal pH of the lipase was determined at a pH ranging between 5.0 and 10.0 at 20 °C and maximal activity was observed at pH 10.0 for *K. marxianus* 83F and *K. ohmeri* 88A (0.02 ± 0.00 $U \cdot mL^{-1}$) (Fig. 3B). Maximal lipase activity was observed at 20 °C in pH 6.0 whereby *K. marxianus* 83F achieved the highest lipase activity (0.06 ± 0.04 $U \cdot mL^{-1}$) followed by *K. ohmeri* 88A (0.03 ± 0.01 $U \cdot mL^{-1}$) and *D. hansenii* 28.12 (0.01 ± 0.00 $U \cdot mL^{-1}$). These results are consistent with the specific growth rate of these strains in olive oil medium as *K. marxianus* 83F showed the highest specific growth rate followed by *K. ohmeri* 88A and *D. hansenii* 28.12. *K. ohmeri* is an oleaginous yeast species (Kitcha & Cheirsilp, 2011) whose strain HB55, isolated by Bussamara et al. (2010), produced lipase with an activity of 0.00324 $U \cdot mL^{-1}$. Out of 125 *K. marxianus* isolates from

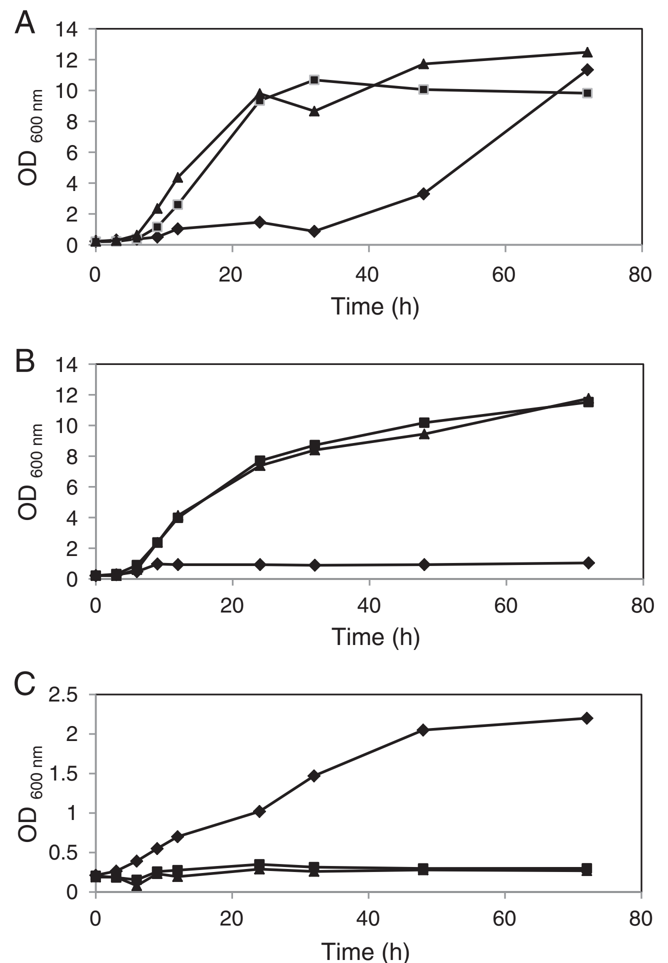

Fig. 2. Yeast growth for 72 h in olive oil medium (A), in lactose medium (B) and in casein medium (C) at 20 °C, and initially, pH 6.0. (A) *Debaryomyces hansenii* 28.12 (♦); *Kluyveromyces marxianus* 83F(■); *Kodamaea ohmeri* 88A (▲); (B) *Debaryomyces hansenii* 28.12 (♦); *Kluyveromyces marxianus* 60P(■); *Kodamaea ohmeri* 88A (▲). (C) *Debaryomyces hansenii* 28.12 (♦); *Kluyveromyces marxianus* 93K(■); *Kodamaea ohmeri* 33H (▲).

Pecorino di Farindola cheese, only 10 isolates showed lipolytic activity, according to Tofalo et al. (2014). The evaluation of enzymatic activity conducted by Padilla, Manzanares, and Belloch (2014) showed that 86%, 91% and 58% of *D. hansenii* isolates of ewe and goat cheeses produced in a small traditional dairy in Mediterranean Spain were able to hydrolyze palmitic, stearic and oleic acid esters, respectively. According to Fleet (2011), the occurrence of *D. hansenii* in dairy products is favored by the production of extracellular proteases and/or lipases.

Intracellular β-galactosidase activity was measured after growth of *K. marxianus* 60P, *D. hansenii* 28.12 and *K. ohmeri* 88A in a lactose (pH 6.0) at 20 °C for 72 h. The temperature and pH profile of

Table 3
Specific growth rates of the yeasts cultivated in olive oil, lactose or casein as inductors, at 20 °C and initially, at pH 6.0.

Medium	Strains	Growth rate (h^{-1})
Olive oil	*Debaryomyces hansenii* 28.12	0.05 ± 0.00
	Kluyveromyces marxianus 83F	0.13 ± 0.00
	Kodamaea ohmeri 88A	0.08 ± 0.00
Lactose	*Debaryomyces hansenii* 28.12	0.14 ± 0.00
	Kluyveromyces marxianus 60P	0.10 ± 0.01
	Kodamaea ohmeri 88A	0.11 ± 0.01
Casein	*Debaryomyces hansenii* 28.12	0.10 ± 0.01
	Kluyveromyces marxianus 93K	0.02 ± 0.00
	Kodamaea ohmeri 33H	0.02 ± 0.02

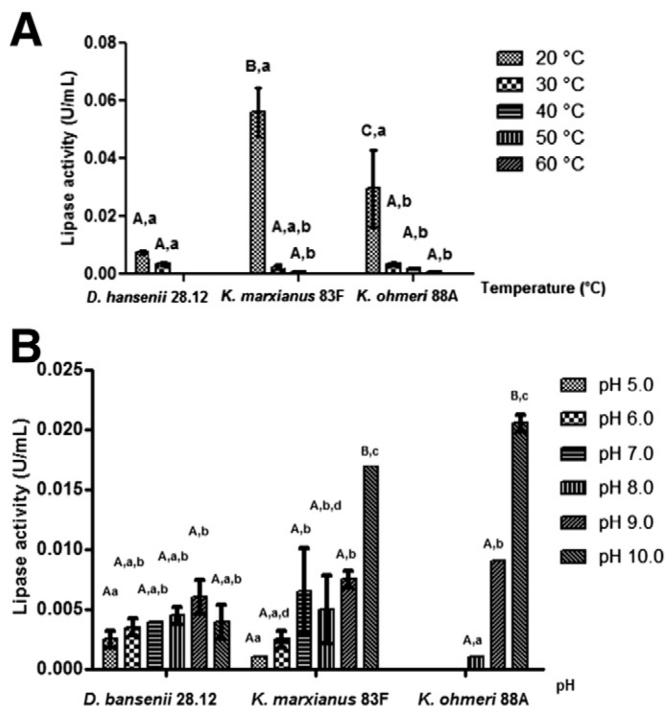

Fig. 3. Lipase activity of the three yeast species most prevalent on Serro Minas cheese at various temperatures at pH 6.0 (A) and at different pH values at 20 °C (B). [A,B,C]At the same temperature and pH, different superscript uppercase letters denote significant differences ($p < 0.05$) among the various yeast species (Bonferroni test). [a, b]Within the same yeast species, different superscript lowercase letters denote significant differences ($p < 0.05$) among the range of temperatures and pH (Bonferroni test).

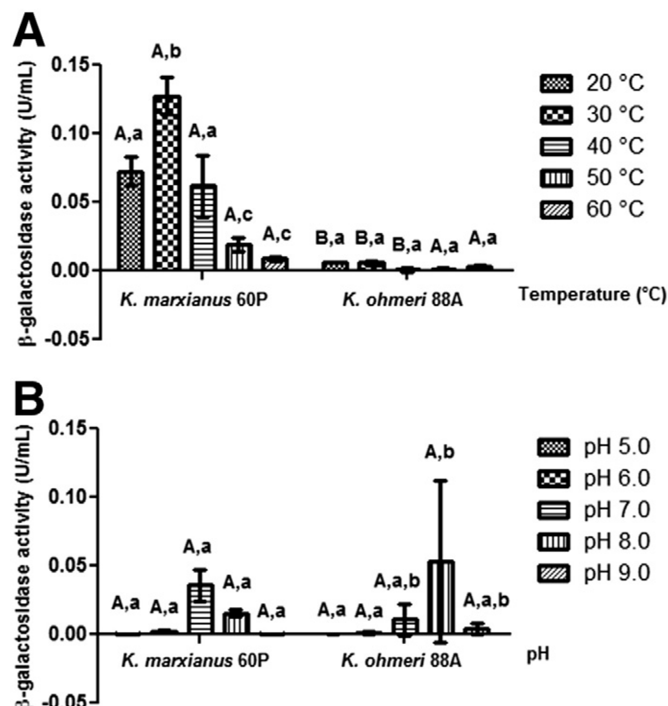

Fig. 4. Temperature and pH profiles from *Kluyveromyces marxianus* 60P and *Kodamaea ohmeri* 88A after 72 h of induction in lactose medium. ß-galactosidase activity in a range of temperatures at pH 7.0 (A) and at different pH values at 30 °C (B). [A,B]At the same temperature and pH, different superscript uppercase letters denote significant differences ($p < 0.05$) among the various yeast species (Bonferroni test). [a, b, c]Within the same yeast species, different superscript lowercase letters denote significant differences ($p < 0.05$) among the range of temperatures and pH (Bonferroni test).

intracellular β-galactosidase activity was carried out from 20 to 60 °C at pH 7.0, and pH 5 to 9 at 40 °C. The maximum β-galactosidase activity produced by *K. marxianus* 60P was 0.13 ± 0.01 U·mL^{-1} at 30 °C (Fig. 4A), with over 55% of this activity at 20 °C. However, the β-galactosidase activity decreased significantly above 20 °C, whereas the optimal temperature for β-galactosidase production by *K. ohmeri* 88A was 0.01 ± 0.00 U·mL^{-1} at 30 °C (Fig. 4A). The maximum β-galactosidase activity was achieved at a pH 7.0 for *K. marxianus* 60P and *K. ohmeri* 88A (Fig. 4B). *D. hansenii* 28.12 did not display any β-galactosidase activity. According to Breuer and Harms (2006), *D. hansenii* has the ability to assimilate but not to ferment lactose. Additionally, Tofalo et al. (2014) demonstrated that from 125 *K. marxianus* isolates of Pecorino di Farindola cheese, all the isolates assimilated lactose. *K. marxianus* has applications in the biotechnology industry, mainly due to its production of β-galactosidase enzyme coupled with high growth rates (Lane & Morrissey, 2010). In this study *K. marxianus* 60P reached a high cell density after 72 h.

Protease activity was determined after growth of *D. hansenii* 28.12, *K. marxianus* 93K and *K. ohmeri* 33H in casein (pH 6.0) at 20 °C for 72 h. *K. marxianus* 93 K and *K. ohmeri* 33H did not achieve good growth in casein (Fig. 2C) and did not show protease activity. Only *D. hansenii* 28.12 demonstrated protease activity and a high growth rate in casein medium. The pH and temperature profile of protease activity were conducted from pH 5 to 9 and 20 °C to 60 °C, respectively (Fig. 5). The extract protease showed maximum activity at 40–50 °C (33.57 ± 2.64 U·mL^{-1} and 34.29 ± 2.17 U·mL^{-1}), and there was no significant difference between the means ($p < 0.05$) (Fig. 5A), with over 40% of this activity occurring at 30 °C. However, the protease activity decreased significantly below 30 °C and above 50 °C. The protease showed maximal activity at pH 8.0 (32.43 ± 2.69 U·mL^{-1}) (Fig. 5B), with over 60% of its activity in a pH range 6–7 and 9–10, significantly decreasing in pH more acidic. The evaluation of enzymatic activity conducted in various studies shows that most *D. hansenii* isolates were able to hydrolyze casein. Proteolytic activity was remarkable in *D. hansenii*, representing more than 90% of the total activity measured as the number of positive isolates of ewe and goat cheeses (Padilla et al., 2014). According to Wojtatowicz, Chrzanowska, Juszczyk, Skiba, and Gdula (2001) approximately 90% of *D. hansenii* (= *Candida famata*) strains isolated from Rokpol cheese demonstrated proteolytic activity < 50 U·mL^{-1} at pH 3.0 and 7.5. Higher proteolytic activity (>100 U·mL^{-1}) was noticed for 12% of the strains with casein at alkaline pH. In this study, protease also showed maximum activity at alkaline pH.

Considering that the mean temperatures during Serro Minas cheese storage vary between 10 °C and 30 °C and that pH values vary between 5.0 and 6.0 (data not shown), the enzymes produced by *K. ohmeri*, *K marxianus* and *D. hansenii* isolates would display high activity during the storage of the cheese because lipase, β-galactosidase and protease showed high activity at 20 °C (pH 6.0), 30 °C (pH 7.0) and 30 °C (pH 6.0), respectively.

Therefore, in this study *D. hansenii* 28.12 showed low lipolytic activity and high proteolytic activity. *K. marxianus* 83F demonstrated lipolytic activity and *K. marxianus* 60P showed β-galactosidase activity. *K. ohmeri* 88A displayed low lipolytic and β-galactosidase activity. Whereas some of the main mechanisms by which yeasts affect cheese ripening include the fermentation of lactose, utilization of lactic acid (with a consequent pH increase), proteolytic and lipolytic activity, and the release of autolytic compounds (Gardini et al., 2006), *D. hansenii* 28.12, *K. ohmeri* 88A and *K marxianus* 60P may be used as starter cultures in Serro Minas cheese because together, they demonstrate proteolytic, lipolytic and β-galactosidase activity.

4. Conclusion

This study provides evidence for the remarkable diversity in yeast communities associated with traditional Serro Minas cheese. The number of yeast species found in cheese is not influenced by the seasons but rather by the duration of the maturation period. The three yeast species

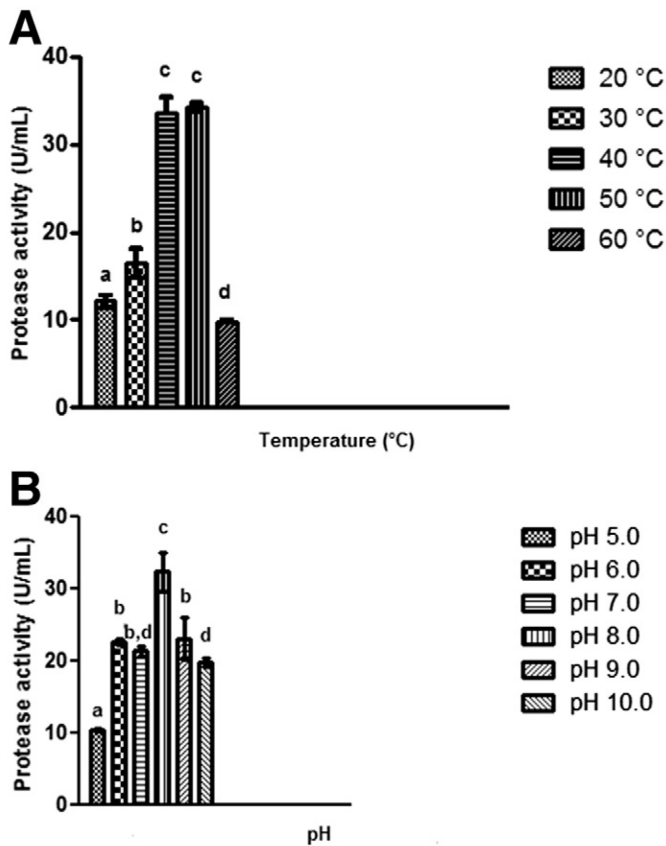

Fig. 5. Temperature and pH profiles from *Debaryomyces hansenii* 28.12 after 72 h of induction in casein medium. Protease activity at various temperatures at pH 6.0 (A) and at different pH values at 40 °C (B). $^{a, b, c, d}$Different superscript lowercase letters denote significant differences ($p < 0.05$) among the range of temperatures and pH (Bonferroni test).

that are predominant in Serro Minas cheese were *D. hansenii*, *K. ohmeri* and *K. marxianus*. These yeast species might be important for the development of cheese flavor during ripening. Because of the protease, lipase and β-galactosidase activity exhibited together by *D. hansenii* 28.12, *K. ohmeri* 88A and *K. marxianus* 60P, these yeast species could represent good starter cultures in the production of Serro Minas cheese.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2014.12.040.

Acknowledgments

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