



# Yeasts from Canastra cheese production process: Isolation and evaluation of their potential for cheese whey fermentation



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## ABSTRACT

Canastra cheese is a cheese with geographical indication recognized by the Brazilian National Institute of Industrial Protection under number IG201002. It is produced in seven municipalities in the state of Minas Gerais in a region called Serra da Canastra. In this work, samples of milk, “pingo” (natural starter), whey and Canastra cheese were collected on a farm in Medeiros-MG/Brazil to evaluate the yeast microbiota and select yeasts for whey fermentation to produce ethanol and volatile aromatic compounds of relevance in the production of cheese. Thirty-nine isolates capable of fermenting lactose in a synthetic medium were identified by MALDI-TOF as *Kluyveromyces lactis* (29), *Torulapora delbrueckii* (7) and *Candida intermedia* (3). Eleven isolates of *K. lactis* and three of *T. delbrueckii* efficiently fermented lactose until 4th day, and due to this reason were selected for cheese whey fermentation with Brix 12, 14 and 18. Generally, the isolates *T. delbrueckii* B14, B35, and B20 and *K. lactis* B10 were the most effective regardless of the initial Brix value. The identification of these four isolates by MALDI TOF was confirmed by sequencing of the ITS region. In the fermentation of cheese whey 14 Brix, *T. delbrueckii* B14 and B35, respectively yielded 24.06 g/L and 16.45 g/L of ethanol, while *K. lactis* B10 was more efficient in the consumption of lactose. In sequential culture with *K. lactis* B10 inoculated 48 h after *T. delbrueckii* B14, 97.82% of the total sugars were consumed resulting in the production of 19.81 g/L ethanol and 39 aromatic volatile compounds. The most abundant compounds were 3-methyl-1-butanol, octanoic acid and ethyl decanoate, which are reported as important for the aroma and flavor of cheeses. Based in our results, B10 isolate inoculated 48 h after B14 isolate is a promising yeast inoculum to be used for fermentation of dairy substrates.

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## 1. Introduction

Canasta cheese is a type of white cheese, produced in Serra da Canastra region, specifically in seven municipalities of Minas Gerais state - Brazil. The geographical indication has been recognized by the National Institute of Industrial Protection (INPI) in 2011 under number IG201002. The climate, altitude, native pasture and Canastra waters (Resende et al., 2011) allow this cheese to have a unique flavor being considered dense and full-bodied. This cheese is produced from cow raw milk inoculated with the commercial rennet and “pingo”. The “pingo” is a type of natural starter obtained from the cheese whey released in cheese production on the previous day. Its microbiota consists of bacteria, particularly lactic acid bacteria, and yeasts (Lima, Lima, Cerqueira, Ferreira, & Rosa, 2009; Nóbrega et al., 2008). Among the yeast species found in the “pingo” and Canastra cheese, Borelli, Ferreira, Lacerda, Franco, and Rosa (2006) reported as the most frequent

*Kodamaea ohmeri*, *Debaryomyces hansenii*, *Torulapora delbrueckii* and *Kluyveromyces lactis*.

Several studies have reported the presence of yeast in milk and its derivatives (Borelli et al., 2006; Lopandic, Zelger, Bănszky, Eliskases-Lechner, & Prillinger, 2006), as they have in their constitution proteins, lipids and organic acids, which favor the growth of various species of yeast (Lopandic et al., 2006). Due to their characteristics, such as production lipolytic and proteolytic activities, assimilation and fermentation of lactose, tolerance of high salt concentrations, low pH, low water activity and low temperatures (Jakobsen & Narvhus, 1996; Lopandic et al., 2006), yeasts isolated from the cheese production process have been studied for their biotechnological potential such as the production of enzymes (Borelli et al., 2006), flavors (Chen et al., 2012) and cheese ripening (Gardini et al., 2006).

The Brazilian production of cheese whey in 2010 Brazil was estimated by Alves et al. (2014) in 8 billion liters. As cheese whey has about 55% of the nutrients contained in milk, it is an interesting source of nutrients for the use in microorganism growth. However, this by-product also has high pollution potential due to its high BOD (Biological Oxygen Demand) and lactose content. Over the years, fermentative processes

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have been considered as an alternative to the use of this by-product, being lactose utilized by various microorganisms such as yeasts and bacteria in order to generate added value products (Becerra, Cerdán, & González-Siso, 2015) such as ethanol (Ozmihci & Kargi, 2008), butanol (Becerra et al., 2015), bacteriocins (Schirru et al., 2014), hydrogen and methane (Cota-Navarro, Carrillo-Reyes, Davila-Vazquez, Alariste-Mondragón, & Razo-Flores, 2011) and  $\beta$ -galactosidase (Rech, Cassini, Secchi, & Ayub, 1999). Among the used microorganisms, *K. lactis* has been the most often studied, mainly in the fermentation of cheese whey to produce ethanol (Christensen, Kádár, Oleskiewicz-Popiel, & Thomsen, 2011) and ethyl acetate (Urit, Löser, Wunderlich, & Bley, 2011).

Recently, the Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF MS) has been used to identify yeasts in fermentative processes for the production of cachaça (Amorim, Schwan, & Duarte, 2016) ethanol, baking, wine and beer (Moothoo-Padayachie, Kandappa, Krishna, Maier, & Govender, 2013; Spitaels et al., 2014). This technique is characterized by its low cost, time consumption and reliability. Although there are works reporting the use of this technique for yeast identification in aforementioned fermentative processes, to the best of our knowledge, there are no published works on the use MALDI TOF MS related to yeasts identification in cheese production process.

Despite reports on the literature regarding the isolation and identification of microorganisms from cheese production process and the use of yeast such as *K. lactis* for cheese whey fermentation, in this work, we report for the first time the use of yeasts isolated from the Canastra cheese production process in a mixed inoculum to ferment cheese whey and produce ethanol and volatile aromatic compounds. Therefore, the objectives of this study were to isolate and identify yeasts from milk, pingo, Canastra cheese and its whey, and also to evaluate the potential of these yeasts in single and mixed culture for the fermentation of cheese whey sugars to produce ethanol and volatile aromatic compounds.

## 2. Material and methods

### 2.1. Sampling and yeasts isolation

Samples of milk, pingo, Canastra cheese and cheese whey were collected in a farm located in the Serra da Canastra region, city of Medeiros - MG/Brazil. The yeast isolation was performed according to the methodology described by Borelli et al. (2006) using YPD medium. After incubation at 28 °C for 48 h, the number of colony forming units (CFU) was recorded and the colonies were properly characterized following the protocol described by Kurtzman, Fell, Boekhout, and Robert (2011). The morphological characteristics of each colony morphotype were recorded and a number of colonies corresponding to the square-root of the number of colonies of each type were re-streaked and purified on YPD. The purified and characterized cellular morphotypes were stored in glycerol 40% at -20 °C for use in later stages of the work.

### 2.2. Lactose fermentation in synthetic medium

The isolates obtained from milk, pingo, Canastra cheese and cheese whey samples were evaluated for their lactose fermentation capacity according to the methodology described by Kurtzman et al. (2011). Briefly, the fermentation basal medium composed by 4.5 g/L of yeast extract and 7.5 g/L of peptone was added of bromothymol blue at a final concentration of 25.6 mg/L of medium. Two milliliters of basal medium was added to a tube containing inverted Durham tube and sterilized for 15 min at 121 °C. After autoclaving, 1 mL of filter sterilized lactose solution was added to a final concentration of 2% (w/v). The fermentations were carried out in triplicate.

### 2.3. Yeasts identification by MALDI TOF

The yeast isolates which fermented lactose in synthetic medium were subjected to MALDI TOF analysis for the identification according to the methodology described by Amorim et al. (2016). All extractions were performed in triplicate and each repetition was spotted three times on the MALDI stainless steel target. The identification of yeast was performed using Biotyper library. According to the manufacturer, score values should be above 1.99 for a reliable identification to the species level. For cluster analysis, the raw spectra were converted into text files using FlexAnalysis software (version 3.4) containing the list of peaks ( $m/z$ ) and their intensities. Spectra were then attenuated, the base line was subtracted and the signal intensities were normalized using the mMass software version 5.5 (Niedermeyer & Strohm, 2012). After treatment of the raw spectra, average spectra were generated and peak picking was performed using a signal-to-noise ratio threshold of 5. The peaks were aligned by generating a consensus peak list using SPECLUST (Alm et al., 2006), available at <http://co.bmc.lu.se/speclust/cluster.pl>. This consensus peak list was then used for the cluster analysis in XLstat 2014.5 software (Addinsoft's, New York, NY) using Pearson similarity and Unweighted Pair-Group Average (UPGA).

### 2.4. Cheese whey fermentation

The yeasts isolates which fermented lactose in synthetic medium until the fourth day were evaluated for their ability to ferment cheese whey with different Brix, 12, 14 and 18. The 12 Brix cheese whey was obtained from the dilution of 14 Brix cheese whey with sterile distilled water, while the 18 Brix cheese whey was obtained by evaporation of 14 Brix cheese whey. Before yeasts inoculation the cheese whey was pasteurized by direct steam in an autoclave for 7 min. The isolates were reactivated in liquid YPD during 24 h/28 °C. After, the biomass was centrifuged at 25 °C/7000 rpm for 10 min to remove the supernatant and washed 2 times with sterile 0.1% peptone water. All isolates were inoculated with a population of  $10^7$  cells/mL. The Brix was measured every 12 h until stabilization.

### 2.5. Confirmation of the yeast identity by sequencing the ITS region

The four yeast isolates that showed better results in cheese whey fermentation under different Brix were subjected to analysis of ITS region to confirm the identity obtained in MALDI TOF analysis. The isolates were reactivated in YPD for 24 h/28 °C and then transferred to a plate containing solid YPD for 24 h/28 °C. A colony was suspended in 1 mL sterile Milli-Q water and centrifuged at 12000 rpm for 1 min and the supernatant was removed. The precipitate was used for DNA extraction using a QIA amp DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA purification was performed on QIA quick PCR Purification Kit (Qiagen). The PCR analysis was performed as described by Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999) and Naumova, Ivannikova, and Naumov (2005) using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The sequencing was performed in the Síntese Biotecnologia Company (Belo Horizonte - MG/Brazil). The obtained sequences were compared with those available in Genbank for subsequent yeast identification. The identification obtained from the sequencing was compared with the identification from MALDI TOF for confirming the identity of the studied yeasts isolates.

### 2.6. Assessment of the yeasts potential to produce ethanol and volatile aromatic compounds

#### 2.6.1. Fermentation for ethanol production using single species inocula

The most efficient yeasts in the fermentation of cheese whey with different Brix were first evaluated as single species inocula for lactose, galactose and glucose consumption and ethanol production. For this

evaluation, experiments were performed with pure cultures of the previously selected yeasts inoculated ( $10^7$  cells/mL) in 14 Brix cheese whey. The inocula multiplication, cheese whey pasteurization, inoculation procedure and fermentation conditions were the same as described in Section 2.4.

### 2.6.2. Fermentation for ethanol and aromatic volatile compounds production using mixed inocula

After the evaluation of yeast fermentative potential in single cultures, those isolates with best results were selected for use in co-cultivation or inoculated sequentially. In co-cultures, two yeasts were inoculated together at the beginning of fermentation, while in the sequential inoculation, one yeast species was inoculated at the beginning of the fermentation and the second species after 48 h of fermentation with the first one. In both cases the used inoculation ratio was 1:1.

After a first fermentation where the mixed inoculum with the highest consumption of glucose, galactose, and lactose and ethanol production was selected, a second fermentation with 1 L of cheese whey was performed. All the fermentations were conducted at 30 °C with the both yeasts of mixed inocula being inoculated in populations of  $10^7$  cells/mL in 14 Brix cheese whey. In both experiments, the kinetic parameter ethanol yield ( $Y_{p/s}$ ), sugars conversion into ethanol efficiency ( $E_f$ ), the total sugar conversion ( $Conv.$ ) and ethanol productivity ( $Q_p$ ) were calculated as described by Duarte et al. (2010a) and Oliveira, Rosa, Morgano, and Serra (2004).

$$[Y_{p/s} = (P_f - P_i) / (S_i - S_f)]; [Q_p = (P_f - P_i) / t_f]; E_f = [(Y_{p/s} * 100) / 0.51]; Conv = [(S_i) / (S_i - S_f) * 100]$$

where  $P_i$  is the initial concentration of ethanol,  $P_f$  is the ethanol concentration at the end of fermentation,  $S_i$  is initial substrate concentration,  $S_f$  is substrate concentration at the end of fermentation.

Ethanol and sugars were analyzed by HPLC and volatile aromatic compounds by GC–MS. All fermentations were carried out in triplicate.

### 2.7. Liquid chromatography analysis

HPLC analysis was performed using a Shimadzu chromatograph, (Shimadzu Corp., Japan) equipped with a refractive index detector (RID-10A) and Supelcogel 8H (Supelco, Bellefonte, PA, USA) column (7.8 mm × 30 cm) operated at 30 °C. The elution was performed with 5 mM sulfuric acid at a flow rate of 0.5 mL/min. The identification of compounds was performed by comparing the retention times of peaks in samples with those of pure standard injected under the same conditions. The quantification was performed by external calibration method (Duarte et al., 2010b).

### 2.8. HS-SMPE gas chromatography mass spectrometry

The solid phase micro extraction (SPME) of volatile compounds was carried out using 5 mL of fermented cheese whey adding 4-nonanol as an internal standard at a concentration of 125 µg/L. Samples were added to 15 mL vials and the extraction of volatile compounds from the head space (HS) was carried out with a DVB/CAR/PDMS 50/30 µm fiber (Supelco, Bellefonte, PA, USA) for 25 min at 60 °C. After extraction, the fiber was kept for 7 min in the injector for desorption of volatile compounds. The analyses were performed on a GC–MS QP2010SE chromatograph (Shimadzu) equipped with Carbowax column (30 m × 0.20 mm id × 0.25 µm) maintained at 50 °C for 5 min, increased 3 °C/min to 190 °C and maintained for 10 min. The temperature of the injector and detector was 230 °C and the carrier gas (He) was used in a flow of 1.2 mL/min. The identification of the compounds was carried out using the NIST library 2011 and pure standards when available. The concentrations were expressed as equivalents of 4-nonanol (internal standard) (Amorim et al., 2016).

### 2.9. Statistical analysis

The software SISVAR 5.6 (Lavras, MG, Brazil) was used for analysis of variance (ANOVA) and Scott-Knott test.

## 3. Results and discussion

### 3.1. Isolation, fermentation of lactose and yeast identification by MALDI TOF

From the different evaluated samples, in the first step, several colonies morphotypes were characterized (Table 1). After this preliminary characterization, a number corresponding to the square root of the total of each morphotype was used for purification and subsequent microscopic characterization. A total of 145 yeast isolates were found, and of these, 83 were from the pingo, 54 from Canastra cheese and 8 from cheese whey. Among the found morphotypes, those designated as H and I were only found in the pingo; J and K were found only in the cheese whey and, E and G were only found in Canastra cheese samples (Table 1). The distribution of the yeast isolates found here is consistent with that reported by Borelli et al. (2006), where a large number of yeasts was observed in samples of cheese and cheese whey, while in the milk this number was lower. This difference in the distribution of yeasts depending on the samples can be attributed to the physicochemical characteristics of milk, pingo, cheese and cheese whey. According to Addis, Fleet, Cox, Kolak, and Leung (2001), temperature, salt concentration, pH and other microorganisms are factors that interfere or limit the growth of yeasts on cheese. One reason for not detecting yeast in milk samples is the fact the samples were collected right after the milking. Therefore, there was no time for yeast multiplication. Other reasons that we believe have influenced the results are the physical-chemical characteristics of milk and the asepsis in the handling of the milk samples until the beginning of the cheese manufacturing process.

The fermentation of synthetic medium resulted in 39 isolates with the ability to ferment lactose (Table 1). These 39 isolates were subjected to MALDI TOF analysis. According to the cluster analysis (Fig. 1), the isolates were differentiated in three main branches (G1, G2 and G3). Twenty nine isolates identified as *K. lactis* were grouped together in a branch (G1) of the dendrogram. The second branch (G2) was composed by 3 isolates identified as *C. intermedia*. In the third branch (G3), 7 isolates were grouped together and identified as *T. delbrueckii*. Fourteen

**Table 1**

Morphological diversity of yeasts colonies isolated from different samples and their lactose fermentation capacity in synthetic medium.

Morphotype	Samples				Lactose fermentation <sup>a</sup>
	Milk	Pingo	Cheese whey	Canastra cheese	
A	–	15	1	7	2
B	–	34	–	22	25
C	–	16	–	7	5
D	–	14	2	8	6
E	–	–	–	2	0
F	–	1	–	5	0
G	–	–	–	3	0
H	–	1	–	–	1
I	–	2	–	–	0
J	–	–	4	–	0
K	–	–	1	–	0
Total	–	83	8	54	39

The characteristics found in each morphotype were: A, beige, circular, margin entire, smooth, and big; B, beige, circular, smooth, and small.

C, beige, round, smooth, and very small; D, white, irregular, umbonate, dull and small; E, beige, irregular, dull and small.

F, white, irregular, margin lobate, dull and small; G, white, irregular, undulate, and very small; H, white, raised, round, large.

I, white, round, flat, and small; J, beige, round, smooth, with brightness and small; K, beige, round, smooth, with brightness and big.

<sup>a</sup> The data correspond to the number of selected strains of each purified morphotype.

isolates showed intense release of CO<sub>2</sub> until the 4th day of fermentation and among them, 11 isolates were *K. lactis* and 3 *T. delbrueckii*. All identifications were performed using Bruker Biotyper library and the scores ranged from 2.24 to 2.85 for *K. lactis*, 2.04 to 2.25 for *T. delbrueckii*, and from 2.22 to 2.30 for *C. intermedia*. According to the manufacturer, score values above 1.99 allow reliably identification to the species level. These data reinforce the efficiency and feasibility of the MALDI TOF technique for the identification and grouping of yeast from fermentation processes as reported in some papers such as (Amorim et al., 2016) and (Usbeck, Wilde, Bertrand, Behr, & Vogel, 2014).

The yeast species identified in this work are often reported by several authors as predominantly associated with milk and milk products (Borelli et al., 2006; Lopandic et al., 2006; Pereira-Dias, Potes, Marinho, Malfeito-Ferreira, & Loureiro, 2000), including in Canastra cheese, its pingo and whey (Borelli et al., 2006; Nóbrega et al., 2008). As reported in other works (Borelli et al., 2006; Nóbrega et al., 2008), *K. lactis* is frequently isolated from dairy products such as cheeses, which might be due to its capacity of fermenting lactose. Six of the seven isolates of *T. delbrueckii* were found in pingo sample, which is in agreement with Borelli et al. (2006), who reported a greater number of this species in pingo samples. The other identified species, *C. intermedia*, has been reported in different types of cheese such as sheep milk cheese by Pereira-Dias et al. (2000) and Gardini et al. (2006), but not reported in the Canastra cheese, although it has already been identified in other cheeses in Minas Gerais/Brazil, as in Serro cheese by Cardoso et al. (2015). This yeast has a variable capacity to ferment lactose, which can probably interfere in its presence or not in substrates rich in lactose. Some yeast species reported by Borelli et al. (2006) were not found in this work, which could be due to the fact that the identification was performed only for a representative group of isolates capable of fermenting lactose.

### 3.2. Selection of isolates for cheese whey fermentation

Eleven isolates of *K. lactis* and 3 isolates of *T. delbrueckii* that fermented lactose in synthetic medium until the 4th day were used

for the fermentation of cheese whey with 12, 14 and 18 Brix (around 120, 140 and 180 g/L of sugars). The different Brix values were used due to the fact that cheese whey concentration is variable according to the industry in which it is generated. Thus, this assay allowed the verification of the yeast's ability to ferment cheese whey from different industries. Four isolates, *T. delbrueckii* B14, B20 and B35 and *K. lactis* B10 showed higher reductions in the Brix value at the end of fermentation independent of the initial Brix (Fig. 2). These 4 yeast isolates were submitted to molecular identification by sequencing the ITS region. The sequences were deposited in NCBI under accession numbers KY203860 (B10), KY203861 (B14), KY203862 (B20) and KY203863 (B35) (Table 2). Based on the sequencing, the isolates B14, B20 and B35 were identified as *T. delbrueckii* with similarity ranging from 96% to 99% when compared to the Genbank sequences used as reference (Table 2). The isolate B10 was identified as *K. lactis* with 96% of similarity compared to the reference sequence KP219449.1 (Table 2). The identification obtained by ITS region sequencing confirmed the identification by MALDI TOF reinforcing the feasibility of this last technique uses for yeast identification in fermentative processes.

The yeast *K. lactis* B10 showed the largest decreases in Brix degree, which were 7.3, 4.6 and 5.4 for the initial Brix 18, 14 and 12, respectively. Among *T. delbrueckii* isolates, the most efficient was B14 with Brix reductions of 6.8, 4.4 and 5.2, respectively for initial Brix of 18, 14 and 12. The largest reductions in Brix presented by *K. lactis* B10 and *T. delbrueckii* B14 may be due to the more efficient use of monosaccharides (glucose and galactose) present in cheese whey and the higher hydrolysis of lactose. Borelli et al. (2006) reported that among 25 studied isolates of *T. delbrueckii*, 2 were β-galactosidase producers, the enzyme responsible for the breakdown of lactose into glucose and galactose, monosaccharides fermented by this yeast. All three evaluated *T. delbrueckii* isolates showed the capacity to grow in YNB with lactose as sole carbon source, which indicates their ability to produce β-galactosidase.

Since the yeast *K. lactis* is part of the 2% of known yeasts species capable of fermenting lactose (Aktaş, Boyacı, Mutlu, & Tanyolaç, 2006; Fonseca, Heinze, Wittmann, & Gombert, 2008) several authors have

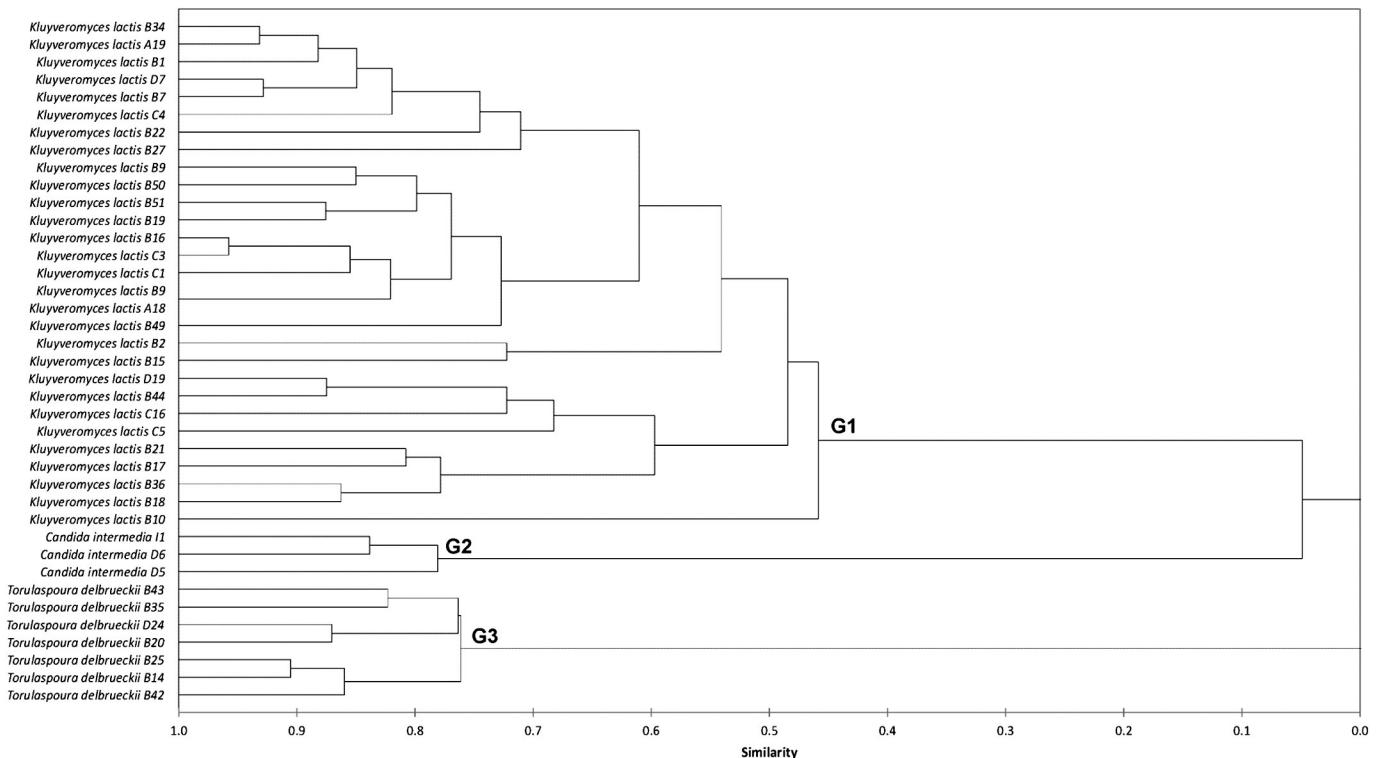


Fig. 1. Dendrogram of protein profile of 39 yeasts isolates that ferment lactose in synthetic medium.



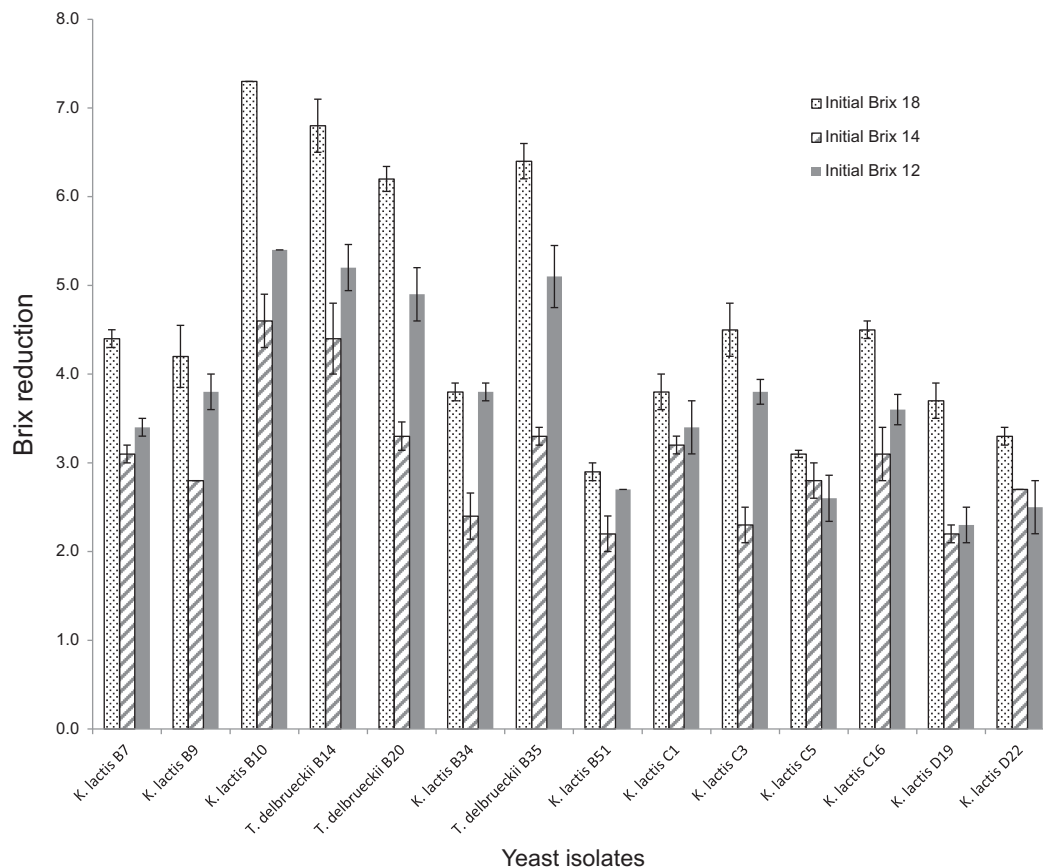


Fig. 2. Profile of sugars consumption (Brix) by different yeasts fermenting cheese whey with three different initial Brix.

isolated this cheese yeast and evaluated its potential for fermentation of lactose and enzyme production (Borelli et al., 2006; Lima et al., 2009; Nóbrega et al., 2008; Padilla, Manzanares, & Belloch, 2014). Although, in general, intermediate reductions was observed for initial 14 Brix, this initial Brix was considered for the fermentations in the next stages of work due to the fact that the cheese whey samples from the supplier industry always presented Brix around 14.

### 3.3. Evaluation of single and mixed inocula for cheese whey fermentation and ethanol production

Initially, the fermentation was carried out with each yeast (*K. lactis* B10 and *T. delbrueckii* B14) inoculated individually for evaluation of sugars consumption and ethanol production (Table 3). The total sugars consumption by *K. lactis* B10 was 98.38 g/L, being 23.33 g/L of lactose, 56.45 g/L of glucose and 18.60 g/L galactose, corresponding to a total conversion of sugars (*Conv*) of 59.22%, ethanol production efficiency (*Ef*) of 25.92% and  $Y_{p/s}$  0.13 g/g (Table 3). Although *K. lactis* B10 has consumed more lactose compared to the other yeast, probably due to its capacity to produce  $\beta$ -galactosidase, this yeast did not show greater

efficiency in ethanol production (Table 3). The highest ethanol production, 24.06 g/L, was observed for *T. delbrueckii* B14 (Table 3). This yeast showed a total sugar consumption of 108.25 g/L being glucose and galactose the main consumed sugars (Table 3). Furthermore, this strain showed *Conv.* value of 65.12%, *Ef* of 43.58% and  $Y_{p/s}$  of 0.22 g/g (Table 3). *T. delbrueckii* B35 have shown the same values of  $Y_{p/s}$  (0.22 g/g), similar values for ethanol concentration and *Conv.*, however, the total sugar consumption (73.81 g/L) presented by this yeast was significantly lower than that observed for *T. delbrueckii* B14.

Taking into account that *K. lactis* B10 was more efficient in the consumption of lactose and the fact that the *T. delbrueckii* B14 showed the highest efficiency in ethanol production and consumption of monosaccharides, both yeasts were selected to be used as mixed inoculum, aiming to optimize the sugar's consumption and ethanol production. In Mix 1, *K. lactis* B10 and *T. delbrueckii* B14 were inoculated together at the beginning of fermentation; Mix 2 was a sequential inoculation of *K. lactis* B10 after 48 h of fermentation with *T. delbrueckii* B14; Mix 3, *T. delbrueckii* B14 was inoculated 48 h after *K. lactis* B10.

As can be seen in Table 4, after 48 h of fermentation with the mixed inoculum of two yeasts, ethanol production was 21.52 g/L, with glucose being completely consumed, while the residual concentration of galactose was 0.48 g/L. However, only after 144 h of fermentation there was significant reduction in lactose content, resulting in total sugars consumption of 106.69 g/L corresponding to a *Conv.* of 92.29%, *Ef* of 38.61% and  $Y_{p/s}$  0.21 g/g (Table 4). The highest total sugars consumption (113.08 g/L) was obtained after 144 h of fermentation when *K. lactis* B10 was inoculated 48 h after *T. delbrueckii* B14 inoculation (Mix 2), resulting in a *Conv.* of 97.82%,  $Y_{p/s}$  0.18 g/g and ethanol concentration of 19.81 g/L ethanol (Table 4). The *Ef* and  $Y_{p/s}$  values found for Mix 1 and Mix 2 after 144 h of fermentation were intermediate values when compared with those obtained for single inocula of *T. delbrueckii* B14 and *K. lactis* B10 (Table 3). However, the total sugar conversion

Table 2  
Yeast species identification by MALDI TOF and ITS region sequencing.

Isolates	Identification		Reference sequence GenBank	Genbank accession number
	MALDI TOF	ITS region		
B10	<i>K. lactis</i>	<i>K. lactis</i>	KP219449.1	KY203860
B14	<i>T. delbrueckii</i>	<i>T. delbrueckii</i>	KU296045.1	KY203861
B20	<i>T. delbrueckii</i>	<i>T. delbrueckii</i>	KF300898.1	KY203862
B35	<i>T. delbrueckii</i>	<i>T. delbrueckii</i>	KU296045.1	KY203863

**Table 3**  
Concentration of sugars and ethanol (g/L) and kinetic parameters for fermentation with yeasts pure cultures.

Samples	Lactose	Glucose	Galactose	Ethanol	Sugars consumption	$Y_{p/s}$ g/g	$E_f$ (%)	Conv. (%)
Cheese whey	60.92 ± 0.81	60.29 ± 0.80	44.91 ± 0.17	nd	–	–	–	–
<i>Torulaspora delbrueckii</i> (B14)	52.87 <sup>b</sup> ± 0.25	nd	5.00 <sup>a</sup> ± 0.23	24.06 <sup>a</sup> ± 0.01	108.25	0.22	43.58	65.16
<i>Kluyveromyces lactis</i> (B10)	37.59 <sup>a</sup> ± 3.59	3.84 <sup>a</sup> ± 4.51	26.31 <sup>b</sup> ± 2.28	13.01 <sup>a</sup> ± 1.15	98.39	0.13	25.92	59.23
<i>Torulaspora delbrueckii</i> (B20)	48.11 <sup>b</sup> ± 0.02	3.62 <sup>a</sup> ± 0.01	28.98 <sup>b</sup> ± 0.04	12.86 <sup>a</sup> ± 0.02	85.41	0.15	29.51	51.41
<i>Torulaspora delbrueckii</i> (B35)	50.42 <sup>b</sup> ± 2.73	10.71 <sup>a</sup> ± 3.51	31.18 <sup>b</sup> ± 4.34	16.45 <sup>a</sup> ± 3.11	73.81	0.22	43.71	44.43

nd - not detected. Values identified by the same letters are not significantly different at the 0.05 level (Scott-Knott test).

(Conv.) was significantly increased due to the consumption of lactose mainly for the Mix 2. The concentration of ethanol and  $Y_{p/s}$  found in this study differ from those reported in the literature, being lower than those reported by Dragone, Mussatto, de Almeida, and Teixeira (2011) or higher than those reported by Ozmihci and Kargi (2008). These differences between the values found here and those reported in the literature can be attributed to cheese whey composition and also to the used yeasts. Although the Mix 1 and Mix 2 have resulted in a similar ethanol concentration after 144 h of fermentation, the total sugar consumption, especially lactose consumption, was higher for Mix 2. This mix was established as best option for cheese whey fermentation to produce ethanol and to be evaluated for the production of volatile aromatic compounds.

#### 3.4. Cheese whey fermentation with selected inoculum for ethanol and aromatic volatile compounds production

Sequential inoculation of *K. lactis* B10 after 48 h of fermentation with *T. delbrueckii* B14 (Mix 2) was repeated fermenting 1 L of 14 Brix cheese whey. The obtained results were similar to those reported previously, being  $Y_{p/s}$  0.14 g/g and  $E_f$  of 27.41%. The differences found between the first and second fermentation can be attributed to the increase in volume of fermented cheese whey, indicating the need for further studies to evaluate other parameters during the fermentation as those reported by Zoppellari and Bardi (2013) including available oxygen and temperature.

The fermentation process generates, in addition to ethanol, a large number of other metabolites such as higher alcohols, esters, acids, ketones and others which can influence the quality of the final product. In this study, 39 aromatic volatile compounds were identified, being 9 acids, 13 alcohols, 13 esters and 4 other compounds (Table 5). The 3-methyl-1-butanol, 2-methyl-1-butanol and 2-phenyl ethanol were the most abundant alcohols being found in concentrations of 123.59 µg/L, 52.72 µg/L and 77.11 µg/L, respectively. In cheeses, 3-methyl-1-butanol is considered a major contributor to the overall flavor of the product. According to Nogueira, Lubachevsky, and Rankin (2005), this compound develops important role in the flavor of Minas cheese. This alcohol is frequently associated with good sensorial descriptors such as “sweet and

fresh”. 2-Methyl-1-butanol is associated with descriptors such as “alcoholic, green and pomace”, while 2-phenylethanol odor is described as “floral” (Curioni & Bosset, 2002). In addition to the presence and importance to cheeses, the alcohols mentioned above were reported by Dragone, Mussatto, Oliveira, and Teixeira (2009) as the main alcohols found in an alcoholic beverage produced from the fermentation of cheese whey.

Hexanoic, octanoic and decanoic were found in concentrations of 107.92 µg/L, 173.04 µg/L, 51.58 µg/L, respectively (Table 5). These three acids together with butanoic acid are frequently reported as presented in cheeses. Moio, Piombino, and Addeo (2000) found that these acids represented approximately 80% of the volatile acid fraction in Gorgonzola cheese. The hexanoic acid and butanoic acid are considered responsible for a “strong and spicy” flavor in cheese (Moio & Addeo, 1998), which may be desirable or not, depending on the type of cheese. The aromatic descriptors of octanoic and decanoic acids are “unpleasant, fatty and rancid” (Curioni & Bosset, 2002).

Production of fatty acids in dairy products may be associated with metabolites generated through lactose metabolism by deamination of amino acids and also by lipid oxidation (Hayaloglu, Brechany, Deegan, & McSweeney, 2008; Nogueira et al., 2005).

Thirteen esters were identified, with a total concentration of 292.09 µg/L. Among them, ethyl decanoate, and 9-ethyl decanoate were found with concentrations of 175.22 µg/L and 62.16 µg/L, respectively (Table 5). Due to their volatility at room temperature, the esters significantly contribute to the taste of many cheeses, even at low concentrations. Besides being associated with descriptors such as “fruity and floral”, these compounds may further reduce the perception of the unpleasant smell of some free fatty acids (Nogueira et al., 2005).

The variety and concentrations of the compounds found in this work indicate that the used yeasts may have potential for use as inoculum in cheese making process during the fermentation or maturation giving improvements in cheese sensory characteristics.

## 4. Conclusions

From a total of 145 isolates, *K. lactis* B10 and *T. delbrueckii* B14, showed better efficiency in fermenting cheese whey when used

**Table 4**  
Concentration of sugars and ethanol (g/L) and kinetic parameters for fermentation with mixed inocula.

Samples	Lactose	Glucose	Galactose	Ethanol	Sugars consumption	$Y_{p/s}$ g/g	$E_f$ (%)	Conv. (%)
Mix 1 48 h	29.19 <sup>c</sup> ± 0.02	nd	0.48 <sup>a</sup> ± 0.08	21.52 <sup>b</sup> ± 0.10	85.93	0.25	49.11	74.33
Mix 1 72 h	22.44 <sup>c</sup> ± 0.90	nd	1.84 <sup>a</sup> ± 0.63	18.86 <sup>b</sup> ± 2.90	91.32	0.20	40.50	79.00
Mix 1 144 h	6.22 <sup>b</sup> ± 3.88	nd	2.68 <sup>a</sup> ± 0.79	21.00 <sup>b</sup> ± 0.01	106.69	0.21	38.61	92.29
Mix 2 48 h	36.36 <sup>d</sup> ± 1.43	nd	0.67 <sup>a</sup> ± 3.61	21.25 <sup>b</sup> ± 4.71	78.56	0.27	53.04	67.96
Mix 2 72 h	1.95 <sup>a</sup> ± 0.68	nd	1.34 <sup>a</sup> ± 0.63	19.71 <sup>b</sup> ± 2.82	112.31	0.18	34.41	97.15
Mix 2 144 h	nd	0.24 <sup>a</sup> ± 1.30	2.28 <sup>a</sup> ± 0.47	19.81 <sup>b</sup> ± 1.02	113.08	0.18	34.35	97.82
Mix 3 48 h	41.62 <sup>e</sup> ± 0.08	22.63 <sup>b</sup> ± 3.97	34.85 <sup>b</sup> ± 0.69	5.21 <sup>a</sup> ± 1.74	59.44	0.09	17.17	37.49
Mix 3 72 h	35.81 <sup>d</sup> ± 0.44	4.21 <sup>a</sup> ± 3.29	33.05 <sup>b</sup> ± 0.38	6.02 <sup>a</sup> ± 0.47	85.47	0.07	13.82	53.91
Mix 3 144 h	30.06 <sup>c</sup> ± 0.34	1.35 <sup>a</sup> ± 2.28	30.43 <sup>b</sup> ± 2.44	9.60 <sup>a</sup> ± 1.53	96.69	0.10	19.72	60.99

Mix 1 - *Torulaspora delbrueckii* B14 and *Kluyveromyces lactis* B10 inoculated together; Mix 2 - *K. lactis* B10 inoculated after 48 h of fermentation with *T. delbrueckii* B14; Mix 3 - *T. delbrueckii* B14 inoculated after 48 h of fermentation with *K. lactis* B10. nd - not detected. Values identified by the same letters are not significantly different at the 0.05 level (Scott-Knott test).

**Table 5**  
Volatile compounds concentration (µg/L) by GC-MS in fermented cheese whey.

N°	Compounds	Concentration
<b>Acids</b>		
1	3-Decenoic acid	0.57 ± 0.00
2	2-Methyl butyric acid*	9.05 ± 1.80
3	Hexanoic acid*	107.92 ± 0.93
4	2-Ethyl caproic acid*	0.97 ± 0.06
5	Heptanoic acid**	1.48 ± 0.60
6	Octanoic acid*	173.04 ± 47.74
7	Nonanoic acid*	1.23 ± 0.53
8	n-Decanoic acid**	51.58 ± 10.63
9	Benzoic acid*	36.58 ± 9.12
	Total	382.44
<b>Alcohols</b>		
10	2-Methyl-1-butanol*	52.72 ± 47.00
11	3-Methyl-1-butanol*	123.59 ± 14.52
12	2-Hexadecanol**	6.96 ± 2.26
13	2-Heptanol*	1.32 ± 0.41
14	2-Ethyl-1-hexanol**	7.90 ± 0.59
15	2-Decanol**	10.46 ± 0.91
16	3-Methyl-2-octanol**	0.28 ± 0.39
17	2,3-Butanodiol**	8.59 ± 0.71
18	5-Ethyl-2-heptanol**	0.23 ± 0.33
19	1-Decanol**	1.74 ± 0.22
20	2-Phenylethanol*	77.11 ± 13.42
21	2-Octanol**	3.97 ± 1.50
22	3-Methyl-2-butanol**	2.51 ± 1.12
	Total	297.39
<b>Esters</b>		
23	Ethyl nonanoate**	7.17 ± 3.41
24	Ethyl 2-hydroxy-4-methylpentanoate**	1.94 ± 0.81
25	Ethyl decanoate*	175.22 ± 37.73
26	Isoamyl octanoate*	0.66 ± 0.31
27	Ethyl 9-decenoate**	62.16 ± 10.83
28	Ethyl dodecanoate**	7.58 ± 1.31
29	Ethyl hexadecanoate**	1.38 ± 0.01
30	Isobornyl acetate**	2.33 ± 0.45
31	Isoamyl lactate*	12.86 ± 1.71
32	Ethyl iso-allochololate**	0.36 ± 0.10
33	cis-3-Decenyl acetate**	1.64 ± 0.41
34	2-Phenethyl acetate*	18.66 ± 2.74
35	trans-9-Tetradecen-1-yl acetate**	0.13 ± 0.03
	Total	292.09
<b>Others</b>		
36	Acetoin*	6.96 ± 2.26
37	Ethylpyrazine*	0.70 ± 0.34
38	Isoborneol**	0.40 ± 0.08
39	Alfa-bisabolol	0.10 ± 0.14
	Total	8.15

Identification based on \*pure standards or \*\*NIST library.

sequentially with *K. lactis* B10 inoculated after 48 h of fermentation with *T. delbrueckii* B14. This mixed inoculum showed potential to produce ethanol from cheese whey sugars, especially lactose, indicating a possible use of this by-product for the production of biofuel. Also, the conversion of cheese whey sugars into aromatic volatile compounds, especially alcohols and esters responsible for pleasant aromas and flavors in cheese, demonstrate that the yeast *K. lactis* B10 and *T. delbrueckii* B14 can be evaluated in the production of cheeses aiming improvements in the sensory characteristics of the final product. Further studies should be performed to optimize the production of ethanol and to evaluate the use of the aforementioned yeast directly in the production of Canastra cheese.

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