RAFAELA PEREIRA ANDRADE

ISOLAMENTO DE LEVEDURAS DO PROCESSO DE PRODUÇÃO DO QUEIJO DA CANASTRA E AVALIAÇÃO DO POTENCIAL DE FERMENTAÇÃO DO SORO DE LEITE

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Prof. Dr. Whasley Ferreira Duarte

Orientador

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ISOLATION OF YEAST IN THE PRODUCTION PROCESS OF CANASTRA CHEESE AND EVALUATION OF THE FERMENTATION POTENTIAL OF WHEY

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Mestre.

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RESUMO GERAL

O queijo canastra é um queijo minas artesanal produzido há mais de 200 anos, na região da Serra da Canastra no estado de Minas Gerais. Esse queijo é produzido a partir de leite cru e inoculado com o coalho comercial e o "pingo". O pingo é um fermento endógeno oriundo da fermentação natural do soro que escorre do queijo nas últimas 24 horas. Os microrganismos presentes no pingo são, em sua maioria, bactérias, especialmente as bactérias do ácido lático, e algumas leveduras. No presente trabalho, objetivou-se isolar e identificar leveduras do pingo, soro, leite e queijo canastra e avaliar sua capacidade de fermentar o soro de leite para geração de diferentes metabólitos, como etanol e compostos aromáticos voláteis. Um total de 145 isolados foram purificados e testados quanto a sua capacidade de fermentar a lactose em meio sintético. Destes, 39 isolados apresentaram capacidade de fermentar a lactose e foram previamente agrupados e identificados por MALDI TOF, sendo identificados como *Kluyveromyces lactis* (29), *Torulaspora delbruckii* (7) e *Candida intermedia* (3). Quatro isolados (*Kluyveromyces lactis* B10, *Torulaspora delbruckii* B14, B20 e B35) apresentaram-se mais eficientes na redução do Brix quando em ensaios de fermentação do soro de leite em três diferentes Brix (18, 14 e 12). A fermentação em soro 14 °Brix com *Torulaspora delbruckii* B14 resultou na produção de 24,06 g/L de etanol, enquanto *Kluyveromyces lactis* B10 consumiu toda lactose do soro. Essas leveduras foram utilizadas em cultivo sequencial com inoculação de *Kluyveromyces lactis* B10 48 h após a inoculação de *Torulaspora delbruckii* B14 resultando na produção de 10,86 g/L de etanol, 0,12 g/g de Yp/s, conversão de açúcares em etanol de 23,14% e conversão total de açúcares de 60,32%. Um total de trinta e nove compostos voláteis aromáticos foram identificados e quantificados na fermentação, sendo os álcoois 2-metil-1-butanol, 3-metil-1-butanol e 2-Feniletanol encontrados nas concentrações de 52,72 µg/L, 123,59 µg/L e 77,11 µg/L respectivamente, 13 ésteres identificados com maiores concentrações para etil decanoato com 175,22 µg/L e acetato de 9-decenoato com 62,16 µg/L, além de 9 ácidos voláteis também identificados na fermentação. Todos os compostos aromáticos são de relevância na produção de produtos lácteos o que, além da produção de etanol indica o potencial dessas leveduras no contexto do presente trabalho.

Palavras-chave: Etanol. Compostos voláteis. Pingo. Soro de leite.

GENERAL ABSTRACT

The Canastra cheese is an artisanal Minas cheese produced for over 200 years in the region of Serra da Canastra, Minas Gerais, Brazil. This cheese is produced from raw milk inoculated with commercial curd and "pingo". "Pingo" is an endogenous leaven derived from natural fermentation of the whey that drains from the cheese in the last 24 hours. The microorganisms present in the "pingo" are mostly bacteria, especially the bacteria from lactic acid and a few yeast. This work had the objective of isolating and identifying yeast from the "pingo", whey, milk and canastra cheese, and evaluating its capacity for fermenting whey to generate different metabolites, such as ethanol and volatile aromatic compounds. A total of 145 isolates were purified and tested regarding their capacity to ferment lactose in synthetic medium. Of these, 39 presented the capacity of fermenting lactose and were previously grouped and identified by MALDI TOF as *Kluyveromyces lactis* (29), *Torulaspora delbruckii* (7), and *Candida intermedia* (3). Four isolates (*Kluyveromyces lactis* B10, *Torulaspora delbruckii* B14, B20 and B35) were more efficient in reducing the Brix when in whey fermentation trials in three different Brix (18, 14 and 12). The fermentation of whey 14°Brix with *Torulaspora delbruckii* B14 resulted in the production of 24.06 g/L of ethanol, while with *Kluyveromyces lactis* B10 all the lactose of the whey was consumed. These yeast were used in sequential cultivation with the inoculation of *Kluyveromyces lactis* B10, 48 hours after inoculation with *Torulaspora delbruckii* B14, resulting in the production of 10.86 g/L of ethanol, 0.12 g/g of Yp/s, 23.14% of sugar conversion into ethanol and 60.32% of total sugar conversion. A total of 39 volatile aromatic compounds were identified and quantified in the fermentation, consisting of alcohols 2-methyl-1-buthanol, 3-methyl-1-buthanol and 2- phenylethanol, in the concentrations of 52.72 µg/L, 123.59 µg/L and 77.11 µg/L, respectively, 13 esters, with higher concentrations for ethyl decanoate, with 175.22 µg/L, and 9-decanoate acetate, with 62.16 µg/L, in addition to 9 volatile acids. All aromatic compounds are relevant in the production of dairy products, which, in addition to the production of ethanol, indicates the potential of these yeasts in the context of this work.

Keywords: Ethanol. Volatile compounds. Pingo. Whey.

SUMÁRIO

CAPÍTULO 1 INTRODUÇÃO GERAL

1 INTRODUÇÃO

A fabricação de queijos artesanais em Minas Gerais vem de uma tradição familiar, passada de pai para filho. Em razão da baixa produção e a dificuldade de transporte do leite a produção de queijo surgiu como uma alternativa para a utilização desse leite. O queijo Canastra é um desses queijos, e as características geográficas da Serra da Canastra fornecem a esse queijo características sensoriais peculiares (INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL, 2016).

O queijo Canastra é o produto típico da região da Serra da Canastra e é considerado o mais importante. Considerado primo distante do queijo Serra da Estrela fabricado em Portugal, é um produto trazido pelos imigrantes da época do ciclo de ouro e produzido no Brasil há mais de duzentos anos. O clima, a altitude e a pastagem nativa da Serra da Canastra fornecem a esse queijo um sabor único, considerado meio picante, forte, denso e encorpado (INPI, 2016). O queijo Canastra é fabricado a partir de leite cru com a adição de coalho comercial e um fermento endógeno chamado “pingo”. O pingo é um fermento líquido obtido do soro liberado pelo queijo do dia anterior, que escorre para um balde, onde permanece por aproximadamente 24 h e é fermentado por sua microbiota natural composta em sua maioria por bactérias, principalmente bactérias do ácido láctico e leveduras (LIMA et al., 2009; NÓBREGA et al., 2008).

O soro de queijo é obtido, por meio da fabricação de queijos, sendo que cerca de 90% do volume de leite utilizado para fabricação de queijo se transforma em soro (DRAGONE et al., 2009a). No Brasil, esse soro é reutilizado na fabricação de novos produtos como achocolatados, ricota, bebidas lácteas, entre outros. Porém, este soro ainda representa um grande problema ambiental,

pois nem todo o soro produzido é reaproveitado, sendo então descartado no meio ambiente de forma incorreta.

Atualmente o reaproveitamento do soro vem sendo realizado buscando principalmente minimizar a poluição ambiental (MAGALHÃES et al., 2011). O soro de queijo tem sido utilizado para a produção de vários compostos, tais como: ácidos orgânicos, etanol, proteína microbiana e vitaminas (OZMIHCI; KARGI, 2008). Esses compostos podem ser utilizados de diferentes maneiras como para a produção de bebidas alcoólicas (GUIMARÃES; TEIXEIRA; DOMINGUES, 2010), para contribuir ao sabor de queijos (álcoois e ésteres) (NOGUEIRA; LUBACHEVSKY; RANKIN, 2005), entre outros.

A fermentação do soro de leite por leveduras, a fim de produzir etanol já vem sendo estudada e utilizada, porém, além do etanol, outros compostos podem ser produzidos, como os compostos aromáticos voláteis de importância para as características do queijo Canastra e que podem ser utilizados de diferentes formas e finalidades. Assim, no presente trabalho, objetivou-se isolar e identificar leveduras presentes no leite, soro, queijo canastra e pingo e avaliar sua aplicação na produção de etanol e compostos voláteis a partir da fermentação do soro de leite.

2 REFERENCIAL TEORICO

2.1 Queijo Canastra

É denominado queijo Canastra o queijo Minas produzido a partir de leite cru na região da Serra da Canastra. A Serra da Canastra é localizada no sudoeste do Estado de Minas Gerais, em região que abriga o Parque Nacional da Serra da Canastra, e é formada por um total de 7 municípios (Figura 1) (EMPRESA DE ASSISTÊNCIA TÉCNICA E EXTENSÃO RURAL, 2004).

Figura 1 - Mapa da localidade e municípios pertencentes a Serra da Canastra.

O queijo Canastra é produzido a partir de leite cru e é fabricado na própria fazenda. O leite é filtrado e então inoculado com coalho comercial e o

"pingo". O pingo é o soro que sai do queijo fabricado no dia anterior que é coletado e é fermentado pelos microrganismos presentes em sua composição. A microbiota do pingo vária de acordo com sua localidade, pois são os microrganismos presentes no leite, soro e equipamentos utilizados é que definem a formação dessa microbiota. Assim, os principais microrganismos encontrados no pingo são as bactérias, principalmente as bactérias do ácido lático, e algumas leveduras, tais como *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Torulaspora delbruekii*, *Candida zeylanoides* e *Kodamaea ohmeri*. (LIMA et al., 2009; NÓBREGA et al., 2008).

Uma hora após a adição do coalho e do pingo a massa é cortada e transferida para formas de plástico, onde serão moldadas e o soro é retirado por pressão manual. O queijo é então coberto com sal por um intervalo de 6 h, invertido e salgado novamente. Após o processo de salga, todo sal é retirado e o queijo é maturado em prateleiras de madeira, a temperatura ambiente. O período de maturação do queijo Canastra pode variar de 3 a 30 dias (BORELLI et al., 2006; LIMA et al., 2009; NÓBREGA et al., 2008). Esse período se estende pelo fato do queijo poder ser consumido fresco, com 3 dias de maturação, ou um pouco mais maturado, com mais dias de maturação, este período de maturação irá depender do gosto do cliente.

2.2 Soro

O soro de queijo é um líquido obtido após a precipitação e a remoção da caseína do leite no processo de fabricação de queijos. Cerca de 90% do volume de leite utilizado para a produção de queijo são transformados em soro, que retém aproximadamente 55% dos nutrientes contidos no leite (DRAGONE et al., 2009a). Os principais nutrientes são: a lactose (45-50 Kg m^{-3}), proteínas e sais minerais (6-8 Kg m^{-3}), lipídios (4-5 Kg m^{-3}) e 8 – 10% de extrato seco. Portanto,

o soro constitui uma matéria- prima barata e de alto teor nutricional, sendo opção para a produção de diferentes compostos (DRAGONE et al., 2011).

O principal problema do soro é o seu potencial poluidor que é aproximadamente 100 vezes maior do que o do esgoto doméstico. Para atender às legislações ambientais, quanto ao descarte incorreto do soro, as indústrias têm buscado alternativas para o seu reaproveitamento. A lactose é a principal responsável pela alta demanda biológica de oxigênio (DQO) do soro e, em decorrência, principalmente, desse fato, vários são os processos fermentativos propostos utilizando o soro para a obtenção de um produto com um maior valor agregado, como: proteínas microbianas, álcoois (etanol, butanol), vitaminas, entre outros (DRAGONE et al., 2009b). Para a obtenção desses produtos de valor agregado é utilizada a lactose do soro de leite fluído ou do soro de leite permeado (concentrado). O soro de leite fluído é o soro obtido diretamente após o processamento do queijo e apresenta menor teor de lactose. Enquanto que o soro de leite permeado contém um teor de lactose em maiores concentrações, pois este sofre um processo de ultrafiltração, onde retira-se água do soro, aumentando a concentração de lactose. A lactose pode ser usada diretamente e consumida por microrganismos ou pode ser pré-hidrolisada e utilizada como substrato para microrganismos lactose negativa, ou seja, microrganismos que não consomem a lactose (GUIMARÃES; TEIXEIRA; DOMINGUES, 2010).

2.3 Leveduras em produtos lácteos

Por apresentar uma boa adaptação a substratos ricos em proteínas, açúcares, lipídeos e ácidos orgânicos, as leveduras são comumente encontradas em produtos lácteos. Outros fatores que auxiliam nessa ampla adaptação são as atividades proteolíticas e lipolíticas desenvolvidas pelas leveduras, sua capacidade de assimilar ou até mesmo fermentar a lactose. Assim, por se tratar de contaminantes naturais, as leveduras podem estar presentes ao longo de toda

a cadeia produtiva do leite, ordenha, transporte e beneficiamento (LOPANDICK et al., 2006).

As leveduras podem exercer funções em produtos lácteos como o de interagir com diferentes microrganismos de diferentes formas, inibindo ou eliminando os microrganismos causadores de defeitos ou patogênicos e podem vir a inibir culturas iniciadoras, ou contribuir de forma positiva aos processos de maturação e fermentação, apoiando essas mesmas culturas (JAKOBSEN; NARVHUS, 1996).

Algumas leveduras já foram isoladas de alguns produtos lácteos como: queijos, coalhada, leite e soro de leite. *Pichia membranifaciens, Saccharomyces cerevisiae* e *Kluyveromyces lactis* foram algumas das leveduras já encontradas em alguns destes produtos (LIMA et al., 2009).

Nóbrega et al. (2008), avaliaram a microbiota leveduriforme do pingo em dois estágios diferentes, no período das águas e da seca, e detectaram 6 espécies diferentes de leveduras em ambos os períodos. Borelli et al. (2006) também avaliaram a microbiota leveduriforme do pingo, juntamente com o soro, leite e queijo canastra, e detectaram 29 espécies de leveduras diferentes. Isso mostra que a presença de leveduras em produtos lácteos, em especial ao queijo e seus derivados, é comum. Essas leveduras podem vir a contribuir de forma favorável com as características sensoriais do queijo, como por meio da produção de enzimas lipolíticas e proteolíticas (BORELLI et al., 2006).

3 CONSIDERAÇÕES GERAIS

No presente trabalho, foram realizados isolamento e identificação de diferentes espécies de leveduras do pingo, soro, leite e queijo canastra. Essas leveduras foram capazes de fermentar o soro de leite, resultando na produção de etanol e compostos aromáticos voláteis de importância econômica e sensorial em queijos. O isoladamente e avaliação do potencial dessas leveduras demonstram a relevância deste trabalho quanto ao aproveitamento do soro de leite e ainda quanto ao papel das leveduras na produção de compostos aromáticos impactantes no aroma e sabor do queijo, a partir da fermentação da lactose, galactose e glicose, açúcares encontrados no leite e soro.

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CAPÍTULO 2 YEASTS FROM CANASTRA CHEESE PRODUCTION PROCESS: ISOLATION AND EVALUATION OF THEIR POTENTIAL FOR CHEESE WHEY FERMENTATION

ABSTRACT

Canastra cheese is a cheese with geographical indication recognized by the National Institute of Industrial Protection under number (IG201002 Brazil). 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 the municipality of Medeiros-MG/Brazil to evaluate the yeast microbiota and after, 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 and sequencing of the ITS region as *Kluyveromyces lactis* (29), *Torulaspora delbrueckii* (7) and *Candida intermedia* (3). Eleven isolates of *K. lactis* and three of *T. delbrueckii* efficiently fermented lactose until day 4, and for this reason were used for whey fermentation with Brix 12, 14 and 18. The isolates *K. lactis* B10 and *T. delbrueckii* B14 were the most effective in reducing Brix regardless of the initial Brix value. In the fermentation of chesse 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 of *T. delbrueckii* B14, 97.82%, the total sugars were consumed resulting in the production of 19.81 g/L ethanol and 39 aromatic volatile compounds. The highest concentrations were found for 3-methyl-1-butanol 123.59 µg/L, octanoic acid 173.04µg/L and ethyl decanoate with 175,22 µg/L. These compounds are reported as important for the aroma and flavor of cheeses.

Key words: Fermentation. Ethanol. Volatile compounds. Lactose. MALDI TOF.

RESUMO

O queijo Canastra é um queijo com indicação geográfica reconhecida pelo Instituto Nacional de Protecção Industrial em número (IG201002 Brasil). É produzido em sete municípios do estado de Minas Gerais em uma região chamada Serra da Canastra. Neste trabalho, amostras de leite, "pingo" (fermento natural), soro de leite e queijo Canastra foram coletados em uma fazenda no município de Medeiros- MG / Brasil para avaliação da microbiota de leveduras e, seleção de leveduras com capacidade de fermentação de soro de leite, com intuito de produzir etanol e compostos aromáticos voláteis de relevância para a produção de queijo. Trinta e nove isolados foram capazes de fermentar a lactose em meio sintético, estes isolados foram identificados por MALDI-TOF e por sequenciação da região ITS, onde, 29 corresponderam a *Kluyveromyces lactis*, 7 a *Torulaspora delbrueckii* e 3 a *Candida intermedia*. Onze isolados de *K. lactis* e três de *T. delbrueckii* foram mais eficientes, fermentando a lactose até o 4° dia de fermentação, e por essa razão foram utilizados para a fermentação de soro de leite com Brix 12, 14 e 18. Os isolados de *K. lactis* B10 e *T. delbrueckii* B14 foram mais eficazes na redução do Brix, independentemente do valor inicial de Brix. Na fermentação do soro de leite , 14 Brix, *T. delbrueckii* B14 e B35, respectivamente, produziram 24,06 g / L e 16,45 g / L de etanol, enquanto *K. lactis* B10 foi mais eficiente no consumo de lactose. Na cultura sequencial com *K. lactis* B10 inoculadas 48 horas depois de *T. delbrueckii* B14, 97,82% dos açúcares totais foram consumidos resultando na produção de 19,81 g / L de etanol e 39 compostos voláteis aromáticos. As concentrações mais elevadas foram encontradas para 3-metil-1-butanol 123,59 g / L, ácido octanóico 173.04 μg / L e decanoato de etilo com 175,22 μg / L. Estes compostos são importantes para o aroma e sabor dos queijos.

Palavras-chave: Fermentação. Etanol. Compostos voláteis. Lactose. MALDI TOF.

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 IG201002 number. The climate, altitude, native pasture and Canastra waters allow this cheese to have a unique flavor being considered medium spicy, strong, dense and full-bodied. This cheese is produced from raw milk inoculated with the commercial rennet and the "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 et al., 2009; NÓBREGA et al., 2008). Among the yeast species found in the "pingo" and Canastra cheese, Borelli et al. (2006) reported the most frequent to be *Kodamaea ohmeri*, *Debaromyces hansenii*, *Toluraposra delbrueckii* e *Kluyveromyces lactis*.

Several studies have reported the presence of yeast in milk and its derivatives, as they have in their constitution proteins, lipids and organic acids, which favor the growth of various species of yeast. These yeasts are reported as producing lipolytic and proteolytic enzymes, are able to assimilate and ferment lactose, tolerate high salt concentrations, low pH, low water activity and low temperatures (JAKOBSE; 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). Among the species often found in the cheese production process, *K.lactis* has been the most extensively used, mainly in the fermentation of cheese whey to produce many valuable products including ethanol and ethyl acetate. Cheese whey originating from the cheese manufacturing process has about 55% of the nutrients contained in milk, which makes ita source of nutrients interesting for

the use in microorganism growth. Over the years, fermentative processes have been presented as an alternative to the use of this by-product, with lactose being utilized by various microorganisms such as yeasts, in order to generate added value products such as ethanol, organic acids, vitamins and microbial proteins (OZMIHCI; KARGI, 2008). However, this by-product also has high pollution potential due to its high BOD (Biological Oxygen Demand) and lactose content.

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* in 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. There fore, the objectives of this study were to isolate and identify yeasts present in 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 MATERIALS 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 yeasts isolation was performed according to the methodology described by Borelli et al. (2006). The plates were incubated at 28 °C for 48h. After incubation, the colonies were properly characterized and evaluated following the protocol described by Kurtzman et al. (2011). Among the different characterized colonies, a number corresponding to thes quare root for each colony morphotype was used for purification and subsequent cells characterization.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).

2.3 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, Shcwan and Duarte (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 version 2.2.For cluster analysis, the row spectra were converted into text files using Flex analysis 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; STROHALM, 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 using Pearson similarity and Unweighted Pair-Group Average (UPGA).

2.4 Cheese whey Fermentation

The yeast 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 cheese whey 12 Brix was obtained from the dilution of 14 Brix cheese whey with sterile distilled water,while the cheese whey 18 Brix was obtained by evaporation of cheese whey 14 Brix.Before yeasts inoculation the cheese whey was pasteurized by direct steam in an autoclave for 7 min.The yeast isolates were reactivated in liquid YPD during 24 h/28 ºC. 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 yeasts that showed better results in cheese whey fermentation under different Brix were subjected to analysis of ITS region to confirm the identity obtained in MALDITOF 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 12,000 rpm for 1 min and the supernatant was removed.The precipitate was used for DNA extraction using a QIAamp DNA Kit (Qiagen, Hilden, Germany)

according to the manufacturer's instructions.DNApurification was performed on QIAquick PCR Purification Kit (Qiagen). The PCR analysis was performed as described by Naumova, Ivannikova and Naumov (2004) 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 Genbankfor subsequent yeasts identification. The identification obtained from the sequencing was compared with the identification from MALDITOF 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

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 (10^7cells/mL) in cheese whey 14 Brix. After the evaluation of yeast fermentative potential in single cultures, those with best results were selected for use in mixed inocula, co-cultivated 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 48h.The fermentations were conducted at 30 °C with the yeasts inoculated in populations of 10^7cells/mL in cheese whey 14 Brix. Samples were collected for determination of ethanol, lactose, galactose and glucose via HPLC. The kinetic parameters ethanol yield ($Y_{p/s}$), sugars conversion efficiency into ethanol (*Ef*), the total sugar conversion

(*Conv.*) and ethanol productivity (Q_p) were calculated as described by Duarte et al. (2010). All fermentations were carried out in triplicate.

2.6.2 Fermentation in bulk - ethanol and volatile aromatic compounds

After choosing the inocula with the highest consumption of glucose, galactose and lactose and most efficient in ethanol production, *T. delbrueckii* B14 and *K. lactis* B10 were used in a sequential inoculation for the fermentation of 1 liter of cheese whey under the same conditions described above. In addition to the samples for HPLC analysis, samples were collected for determination of volatile aromatic compounds by GC-MS. The fermentations were performed in duplicate.

2.6.3 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 X 30 cm) operated at 30 °C. The elution was performed with 5mM 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., 2010).

2.6.4 HS-SMPE Gas chromatography mass spectrometry

The solid phase micro extraction (SPME) of volatiles was carried out using 5 mL sample of fermented cheese whey added to4-nonanol as an internal standard at a concentration of 125 µg/L. Samples were added to vials of 15 mL, and extraction carried out with a DVB/CAR/PDMS 50/30 µm fiber (Supelco, Bellefonte, PA, USA) for 25 min at 60 °C. After extraction of compounds in head space (HS) the fiber was kept for 7 min in the injector for desorption of

volatile compounds. The analyses were performed on a GC-MSQP2010SE chromatograph (Shimadzu) equipped with Carbowax column (30 m X 0.20 mm id X 0.25 um) 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 flow 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; SHCWAN; DUARTE, 2016).

3 RESULTS AND DISCUSSION

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

The different samples were evaluated for their yeast diversity, and inthe 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. After these procedures, 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 morphotypes E and G were only found in Canastra cheese samples (Table 1). The distribution of yeast isolates found here is consistent with that reported by Borelli et al. (2006) where a large number of yeasts in samples of cheese and cheese whey was observed, 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 et al. (2001), temperature, salt concentration, pH and other microorganisms are factors that interfere or limit the growth of yeasts on cheese.

Table 1 - Morphological diversity of yeasts colonies isolated from different samples and their lactose fermentation capacity in synthetic medium.

Morfotipo	Leite	Pingo	Soro	Queijo	Fermentaram lactose
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

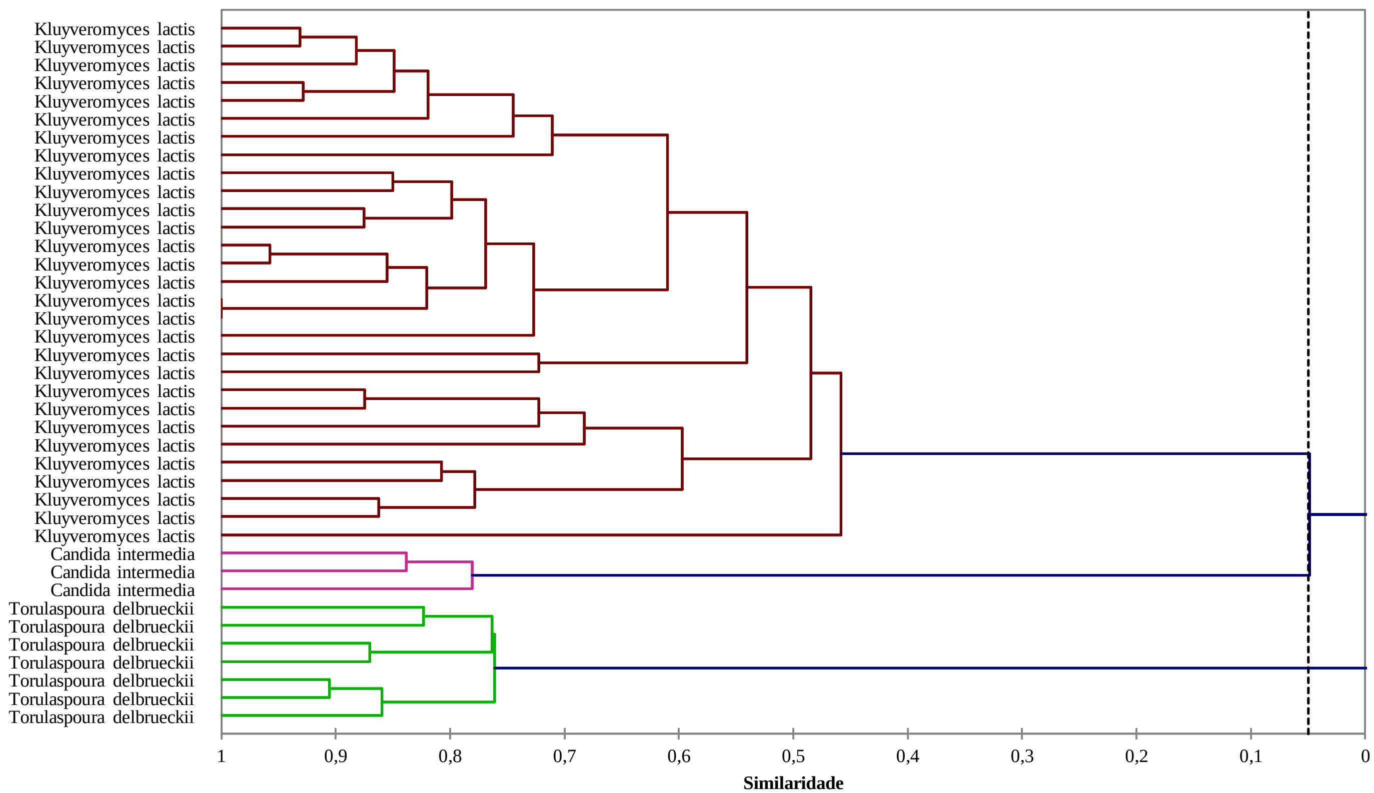
* The data correspond to the number of selected strains of each purified morphotype.
The characteristics found in each morphotype were: A, beige, round, smooth, low gloss and great; B, beige, round, smooth, low gloss and small; C, beige, round, smooth, low gloss and very small; D, white, uneven, rough, dull and small; And, beige, uneven, dull and small; F, white, uneven, rough, dull and small; G, white, uneven, rough, dull and very small; H, white, round, dull and large; I, white, round, flat, dull and small; J, beige, round, smooth, with shine and small; K, beige, round, smooth, with brightness and great.

After macroscopic and microscopic characterization, in the fermentation of synthetic medium, 39 isolates showed the ability to ferment lactose (Table 1). Among then 14 isolates showed intense release of CO_2 until the 4th day of fermentation. These 39 isolates were subjected to MALDI TOF analysis of (Figure 1).According to the cluster analysis (Figure 1), 29 isolates identified as *K. lactis* were grouped in a branch of the dendrogram. In a second branch, 7 isolates were grouped together and identified as *T. delbrueckii*, while the other 3 isolates were identified as *C. intermedia* and grouped distinctly from the others.

Among the 14 yeast isolates with higher efficiency (until 4th day) of lactose fermentation, 11 were *K. lactis* and 3 *T. delbrueckii*. All identifications were performed using Bruker Biotyper 2.2 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, Shcwan, and Duarte (2016) and Usbeck et al. (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; DIAS et al., 2000; LOPANDIC et al., 2006), inclusive in Canastra cheese, its pingo and whey (BORELLI et al., 2006; NOBREGA et al., 2008).The yeast *K. lactis* is one of the known yeast species capable of fermenting lactose, which is one of the reasons for the frequent isolation of this yeast from dairy products such as cheeses. *T. delbrueckii*, as in this work (among 7 isolates, 6 were from pingo), as found by Borelli et al. (2006) in greater numbers in pingo samples. The other identified species, *C. intermedia*, has been reported in different types of cheese such as sheep milk cheese by Dias et al. (2000) and ewe milk cheese by 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).

Figure 1 - Dendrogram protein profile of yeasts which fermented lactose synthetic medium.

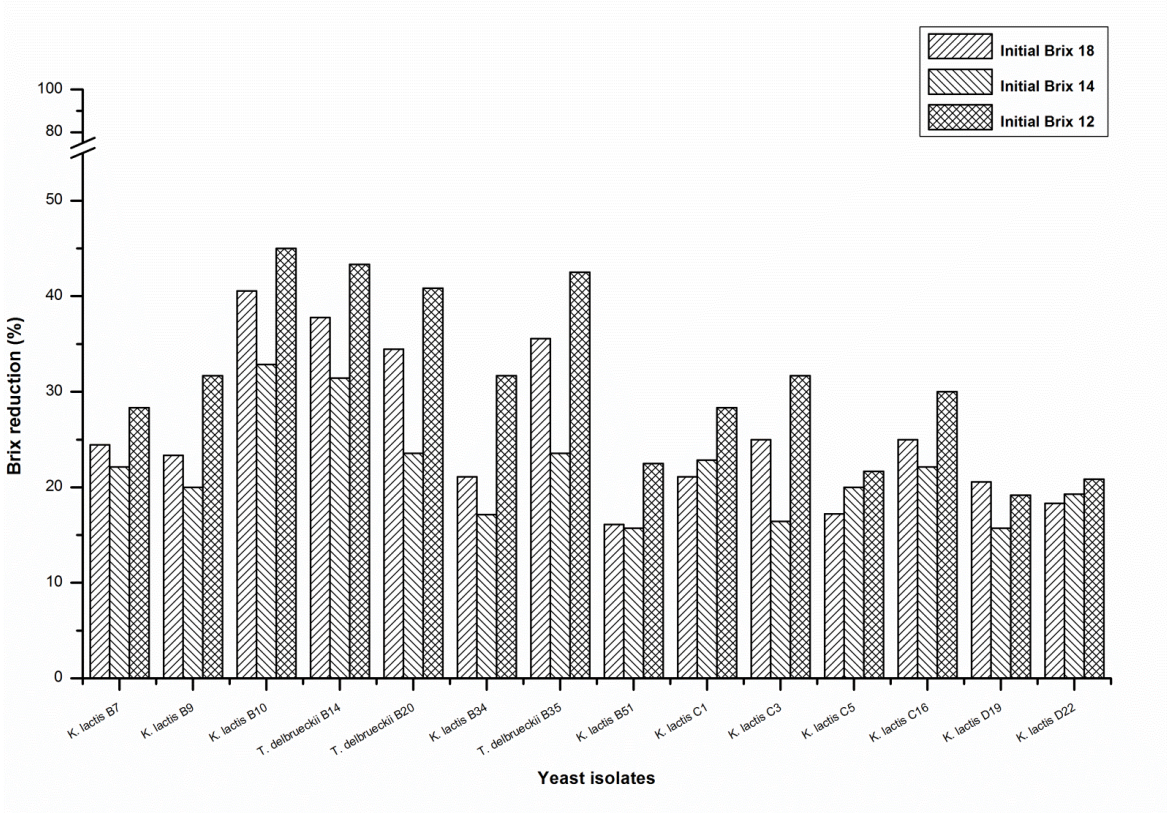


Source: Author's data (2016).

3.2 Selection insulated whey fermentation

The 11 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. 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 yeasts ability to ferment cheese whey from different industries. Four yeasts, *T. delbrueckii* (isolated 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 (Figure 2). The identification by MALDI TOF of these 4 yeast isolates was confirmed by sequencing the ITS region (data not shown).

Figure 2 - Profile of sugars consumption (Brix) for the fermentation of cheese whey with three different initial Brix values.



Source: Author's data (2016).

The yeast *K. lactis* B10 showed the largest decreases in Brix degree, which were 40.56%, 32.86% and 45.00% for the initial Brix 18, 14 and 12, respectively. Among *T. delbrueckii* isolates, the most efficient was B14 with Brix reductions of 37.78%, 31.43% and 43.33% respectively for initial Brix of 18, 14 and 12.The greatest reductions in Brix presented by yeast *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 isolates of *T. delbrueckii* studied, 2 were producers of β-galactosidase, the enzyme responsible for the breakdown of lactose into glucose and galactose, monosaccharides fermented by this yeast. Since the yeast *K. lactis*is is part of the 2% of known species of yeast capable of fermenting lactose (BOYACI; MUTLU; TANYOLAC, 2006; FONSECA et al., 2008) several authors have isolated this cheese yeast and evaluated its potential for assimilation and fermentation of lactose and enzyme production (BORELLI et al., 2006; LIMA et al., 2009; NÓBREGA et al., 2008; PADILLA; MANZANARES; BELLOCH, 2014). Although generally for Brix 14 reductions were observed with intermediate values (%), this initial Brix was considered for the fermentations of the next stages of work due to the fact that the collected cheese whey samplesfrom the supplier industry, always presented Brix around 14.

3.3 Evaluation of yeasts potential for cheese whey fermentation and ethanol production

Initially fermentation was carried out with each yeast (*K. lactis* B10 and *T. delbrueckii* B14) individually for evaluation of sugars consumption and ethanol production (Table 2).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*) 59.22%., ethanol

production efficiency (*Ef*) of 25.92% and $Y_{p/s}$ 0.13 g/g (Table 2).Although *K. lactis* B10 has consumed more lactose compared to the other yeast, probably due to its capacity to produce β-galactosidase, this yeast did not show greater efficiency in ethanol production (Table 2).The highest ethanol production, 24.06 g/L, was observed for *T. delbrueckii* B14 (Table 2).This yeast showed a total sugar consumption of 108.25 g/L being glucose and galactose (Table 2) the main sugars consumed. Fur the rmore, this strain showed *Conv. v*alue of 65.12%, *Ef* of 43.58% ethanol and $Y_{p/s}$ of 0.22 g/g (Table 2).Although *T.delbrueckii* B35 have shown the same values of $Y_{p/s}$ (0.22 g / g),similar values for ethanol and *Conv.* (43.71%), 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, to optimize the sugars consumption, mainly lactose, and production of ethanol.In Mix 1, *K. lactis* B10 and *T. delbruekii* B14 were inoculated together at the beginning of fermentation; Mix 2 was composed of*K. Lactis* B10 inoculated after 48 h of*T. delbrueckii* B14 inoculation; finally, in Mix 3, *T. delbrueckii* B14 was inoculated 48 h after *K. lactis* B10.

Table 2 - Concentration of sugars and ethanol (g / L) and kinetic parameters for yeasts pure cultures.

Samples	**Lactose g/L**	**Glucose g/L**	**Galactose g/L**	**Ethanol g/L**	**Sugarscons umptiong/L**	**Yp/s g/g**	**Ef (%)**	**Conv. (%)**	**Qp g/L.h**
Cheese whey	60, 92 ±0,81	60, 29 ±0,80	44, 91 ±0,17	Nd	-	-	-	-	-
Torulasporadelbruekii(B14)	52, 87 ±0,25	Nd	5, 00 ±0,23	24, 06 ±0,01	108, 25	0, 22	43, 58	65, 16	0, 25
Kluveromyceslactis(B10)	37, 59 ±3,59	3, 84 ±4,51	26, 31 ±2,28	13, 01 ±1,15	98, 39	0,13	25, 92	59, 23	0, 14
Torulasporadelbruekii(B20)	48, 11 ±0,02	3, 62 ±0,01	28, 98 ±0,04	12, 86 ±0,02	85, 41	0, 15	29, 51	51, 41	0,13
Torulasporadelbruekii(B35)	50, 42 ±2,73	10, 71 ±3,51	31, 18 ±4,34	16, 45 ±3,11	73, 81	0, 22	43, 71	44, 43	0,17

Nd- not detected

As can be seen in Table 3, after 48 h of fermentation with the 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 of lactose, resulting in total sugars consumption of 106.69 g/L corresponding to a Conv. of 92.29%,Efof 38.61% and Yp/s 0.21 g/g (Table 3). The highest total sugars consumption (113.08 g/L) was obtained after 144h of fermentation when K. lactis B10 was inoculated 48 h after T. delbrueckii B14 (Mix 2), resulting in a Conv. of 97.82%, Yp/s 0.18 g/g and ethanol concentration of 19.81 g/L ethanol (Table 3).The Ef and Yp/s values found for Mix 1 and Mix 2 after 144 h of fermentation were intermediate values when compared with those obtained for single inoculaof T. delbrueckii B14 and K. lactisB10 (Table 2). However, the total sugar conversion (Conv.) was significantly increased due to the consumption of lactose mainly for theMix 2.The values for concentration of ethanol and Yp/s found in this study differ from those reported in the literature, being lower than those reported by (DRAGONE et al., 2011) or higher than those reported by Ozmihci and Kargi (2008). These differences in values can be attributed to differences in cheese whey composition and also to yeastsused. Although the Mix 1 and Mix 2 have resulted in a similar ethanol concentration after 144 h of fermentation, the total sugars consumption, especially lactose consumption, was higher for Mix 2. This mix was established as best option for cheese whey fermentation to produce ethanol and volatile aromatic compounds.

3.4 Fermentation in bulk

Sequential inoculation *K. lactis* B10 48 h after inoculation *T. delbrueckii* B14 (Mix2) was repeated fermenting 1 L of cheese whey. The obtained results were similar to those reported previously (data not showed). The most significant difference was found for *Ef*, which value was around 10% lower than

the *Ef* reported above. This reduction can be attributedto the increase in volume of fermented cheese whey, indicating the need for evaluation of other parameters during the fermentation as those reported by Zoppellari and Bardi (2013) including available oxygen, temperature and deproteinized cheese whey.

Table 3 – Concentration of sugars and ethanol (g / L) and kinetic parameters for mixed fermentations.

Amostras	Lactose g/L	Glucose g/L	Galactose g/L	Ethanol g/L	Sugarsconsuption g/L	Yp/s g/g	Ef (%)	Conv (%)	Qp g/L.h
Mix1 48h	29,19 ±0,02	Nd	0,48 ±0,08	21,52 ±0,10	85,93	0,25	49,11	74,33	0,45
Mix1 72h	22,44 ±0,90	Nd	1,84 ±0,63	18.86 ±2,90	91,32	0,20	40,50	79,00	0,26
Mix1 144h	6,22 ±3,88	Nd	2,68 ±0,79	21.00±0,01	106,69	0, 21	38,61	92,29	0,15
Mix2 48h	36,36 ±1,43	nd	0,67 ±3,61	21.25 ±4,71	78,56	0, 27	53,04	67,96	0,44
Mix2 72h	1,95 ±0,68	Nd	1,34 ±0,63	19,71 ±2,82	112,31	0, 18	34,41	97,15	0,27
Mix2 144h	Nd	0,24 ±1,30	2,28 ±0,47	19,81±1,02	113,08	0,18	34,35	97,82	0,14
Mix3 48h	41,62 ±0,08	22,63 ±3,97	34,85 ±0,69	5,21 ±1,74	59,44	0, 09	17,17	37,49	0,11
Mix3 72h	35,81 ±0,44	4,21 ±3,29	33,05 ±0,38	6,02 ± 0,47	85,47	0, 07	13,82	53,91	0,08
Mix3 144h	30,06 ±0,34	1,35 ±2,28	30,43 ±2,44	9,60 ±1,53	96,69	0, 10	19,72	60,99	0,07

Cheese whey values correspond to the 0 h of fermentation. Mix 1- both yeast *Torulasporadelbruekii*B14 and *Kluveromyceslactis*B10, inoculated together; Mix 2 – T.delbruekiiB14 inoculated and after 48 h of fermentation K.lactis B10 was inoculated with Mix 3- K. lactisB10 inoculated and after 48 h of fermentation T.delbruekii B14 was inoculated. nd not detected.

The fermentation process generates, in addition to ethanol, a large number of other metabolites higher in alcohols such as, esters, acids, ketones and others which can influence the quality of the final product. In this study 39 aromatic volatile compounds were identified, with 9 acids, 13 alcohols, 13 esters and 4 other compounds (Table 4).The total concentration of alcohols was 297.39 µg/L and 3-methyl-1-butanol, 2-methyl-1-butanol and 2-phenyl ethanol were the most abundant alcohols being found in concentrations of123.59 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 tastein Minas cheese.This alcohol generates the "sweet and fresh" flavor of soft cheeses (LAW, 1992; NOGUEIRA; LUBACHEVSKY; RANKIN, 2005). In addition to its presence and importance to cheeses, the alcohols mentioned above were reported by Dragone et al. (2009) as the main alcohols found in an alcoholic beverage produced from the fermentation of cheese whey.

Among the acids, hexanoic, octanoic and decanoic were found in concentrations of107.92µg/L, 173.04 µg/L, 51.58 µg/L, respectively (Table 4). The hexanoic acid and butanoic acid are considered responsible for a "strong and spicy" flavor in cheese (MOIO; PIOMBINO; ADDEO, 1999), which may be desirable or not, depending on the type of cheese. Production of fatty acids in dairy products may be associated with metabolites generated through lactose metabolism by desamination of amino acids and also by lipid oxidation (HAYALOGLU et al., 2008, NOGUEIRA; LUBACHEVSKY; RANKIN, 2005).

Thirteen esters were identified, with a total concentration of 292.09 µg/L. Among them, ethyl decanoate, and 9-ethyl decenoatewere found with concentrations of175.22 µg/L and 62.16 µg/L, respectively (Table 4). Because of its volatility at room temperature, the esters contribute significantly to the

taste of many cheeses, even at low concentrations. Besides being associated with aromas "fruity and floral", these compounds may further reduce the perception of the unpleasant smell of some free fatty acids (NOGUEIRA; LUBACHEVSKY; RANKIN, 2005).The variety and concentrations of the compounds found in this work indicate that the yeasts used may have potential for use in cheese making giving improvements in cheese sensory characteristics.

Table 4 - Volatile compounds identified by GC-MS in fermented cheese whey.

(continue)

Nº	**Compounds**	**Concentration µg/L**
	Ácido	
1	3-Decenoic Acid	0,57 ±0,00
2	2-Methylbutyric Acid	9,05 ±1,80
3	Hexanoic Acid	107,92 ±0,93
4	2-Ethylcaproic 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	Decanoic Acid	51,58 ±10,63
9	Benzoic Acid	36,58 ±9,12
	Total	382,44
	Alcohols	
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-Butanediol	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

Table 4 - Volatile compounds identified by GC-MS in fermented cheese whey.

(conclusion)

Nº	**Compounds**	**Concentration µg/L**
	Esters	
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-Allocholate	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
	Others	
36	Acetoin	6,96 ±2,26
37	Ethylpyrazine	0,70 ± 0,34
38	Isoborneol	0,40 ±0,08
39	Alpha-Bisabolol	0,10 ±0,14
	Total	8,15

Source: Author's data (2016).

4 CONCLUSIONS

From a total of 145 isolates, *K. lactis* B10 and *T. delbrueckii*B14, showed better efficiency in fermenting cheese whey when used sequentially with *K. lactis* B10 inoculated 48 h after *T. delbrueckii*B14. The ethanol produced demonstrated the potential of the mixed inoculum in the conversion of cheese whey sugars, especially lactose, indicating a possible use of this by-product for the production of biofuel in order to add value to the productive chain of the Canastra cheese, while minimizing environmental problems caused by cheese whey. 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 used to produce cheeses with the purpose of generating these compounds and consequently improve the sensory characteristics of the final product. Further studies should be performed to optimize the production of ethanol and further evaluate the use of the aforementioned yeast directly in the production of Canastra cheese.

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