UNIVERSIDADE FEDERAL DE LAVRAS

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INDIGENOUS YEAST AS STARTER CULTURES FOR CHEESE PRODUCTION

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Tese 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 Doutor.

Prof. Dr. Whasley Ferreira Duarte

Orientador

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USO DE LEVEDURAS ISOLADAS DA PRODUÇÃO DO QUEIJO CANASTRA COMO CULTURAS INICIADORAS NA PRODUÇÃO DE QUEIJOS

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RESUMO GERAL

A procura por alimentos funcionais acrescidos de microrganismos probióticos vem crescendo consideravelmente no mundo atual, porém há uma dificuldade pelas indústrias alimentares de incorporar o probiótico ao alimento, pois cada microrganismo apresenta uma exigência fisiológica. Portanto o alimento, ao qual será incorporado, deve suprir essa exigência. Dentre os probióticos já comercializados, a maioria são bactérias, sendo reconhecida apenas uma levedura. O objetivo do trabalho foi avaliar o potencial probiótico e tecnológico das leveduras *Kluyveromyces lactis* B10 e *Torulaspora delbrueckii* B14 isoladas do processamento do queijo Canastra e avaliar o seu uso como culturas iniciadoras em queijos. A resistência ao trato gastro intestinal foi avaliada pela passagem pelo trato gastrointestinal em condições simuladas. Outros como de autoagregação, hidrofobicidade, inibição de patógenos e produção de β-galactosidase foram realizados. A caracterização tecnológica foi realizada pela avaliação da capacidade de sobrevivência das leveduras, em diferentes concentrações de NaCl (2,5; 5 e 10%) e, em diferentes temperaturas (4 e 40 °C), por 21 dias. As leveduras foram avaliadas como culturas iniciadoras, simples e mistas na produção de queijos. Os queijos foram analisados via HPLC e GC-MS. Após a passagem pelo trato gastrointestinal simulado, as leveduras *T. delbrueckii* B14 e *K. lactis* B10 apresentaram viabilidade acima de 80%, taxas acima de 90% de autoagregação e produção de 0,35 U/g e 0,53 U/g de β-galactosidase, respectivamente. Ambas as leveduras apresentaram sobrevivência, quando expostas às diferentes concentrações de NaCl e a 4 °C, contudo, quando expostas a 40 °C, a levedura *T. delbrueckii* B14 não apresentou sobrevivência, e *K. lactis* B10 sobreviveu apenas nos sete primeiros dias de avaliação. A lactose presente nos queijos foi parcial ou completamente consumida, mostrando que ambas as leveduras possuem capacidade de degradar a lactose. Um total de 37 compostos voláteis foi encontrado, sendo seis ácidos, nove álcoois, 14 ésteres, três aldeídos e seis outros. Dentre os ácidos, o isocaproico, hexanoico, decanoico e butanoico foram os mais abundantes em todos os queijos. Os álcoois encontrados, em maiores concentrações, foram os 2,3- butanediol, o fenetil álcool, isoamil álcool e isobutanol. E os ésteres isoamil acetato e fenetil acetato foram os mais abundantes em todos os queijos. Os resultados demonstraram que as leveduras *K. lactis* B10 e *T. delbrueckii* B14 apresentam resistência à passagem pelo trato gastro intestinal e podem ser utilizadas, na produção de queijos, como culturas iniciadoras, com produção de compostos aromáticos voláteis que auxiliam na melhoria da qualidade do produto final.

Palavras-chave: Probióticos. Leveduras. Queijos.

ABSTRACT

The demand for functional foods with probiotic microorganisms is currently growing considerably. However, there is a difficulty for the food industries to incorporate the probiotic into the food because each microorganism has a physiological requirement. Therefore, the food to which it will be incorporated must meet this requirement. Among the probiotics already sold, most are bacteria, with only one yeast being recognized. The objective of this work was to evaluate the probiotic and technological potential of *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 yeasts isolated from Canastra cheese processing and to evaluate their use as starter cultures in cheese. Resistance to the gastrointestinal tract was assessed by passage through the gastrointestinal tract under simulated conditions. Others such as self-aggregation, hydrophobicity, pathogen inhibition and β-galactosidase production were performed. The technological characterization was performed through the evaluation of yeast survival capacity at different NaCl concentrations (2.5, 5 and 10%) and at different temperatures (4 and 40 ºC) for 21 days. The yeasts were evaluated as single and mixed starter cultures in cheese production. The cheeses were analyzed via HPLC and GC-MS. After the passage through the simulated gastrointestinal tract, *T. delbrueckii* B14 and *K. lactis* B10 yeasts presented viability above 80%, self-aggregation rates above 90% and yield of 0.35 U/g and 0.53 U/g of β-galactosidase, respectively. Both yeasts presented survival when exposed to different NaCl concentrations and at 4 ºC, but when exposed to 40 ºC *T. delbrueckii* B14 yeast did not survive, and *K. lactis* B10 survived only in the first 7 days of evaluation. The lactose present in the cheeses was partially or completely consumed, showing that both yeasts have the capacity to degrade lactose. A total of 38 volatile compounds were found, 6 acids, 9 alcohols, 14 esters, 3 aldehydes and 6 others. Among the acids, isocaproic, hexanoic, decanoic and butanoic were the most abundant in all cheeses. The alcohols found in higher concentrations were 2,3-butanediol, phenethyl alcohol, isoamyl alcohol and isobutanol. And isoamyl acetate and phenethyl acetate esters were the most abundant in all cheeses. The results show that *K. lactis* B10 and *T. delbrueckii* B14 yeasts are resistant to passage through the gastrointestinal tract and that they can be used in the production of cheese as starter cultures, with the production of volatile aromatic compounds that help improve the final product quality.

Key words: Probiotic. Yeast. Cheese.

SUMÁRIO

1 INTRODUÇÃO

Os probióticos são definidos como microrganismos, que, quando ingeridos vivos, em uma quantidade adequada, conferem benefícios à saúde de seu hospedeiro (FAO/WHO, 2002). Os probióticos são adicionados em uma vasta gama de alimentos, porém são os mais utilizados em alimentos lácteos fermentados. Outra forma de consumo é por meio de suplementos farmacêuticos. Atualmente a maioria dos probióticos são bactérias, tendo apenas uma espécie de levedura, *Saccharomyces cerevisiae var boulardi*, como probiótica (FERNÁNDEZ-PACHECO et al., 2019). Esse fato instiga a pesquisa por novas espécies de leveduras probióticas. Algumas espécies de leveduras vêm sendo estudadas, quanto à sua capacidade probiótica, e autores como Fadda et al. (2017) e Diosma et al. (2014) verificaram o potencial probiótico de algumas leveduras como *Kluyveromyces lactis* e *Saccharomyces cerevisiae*.

O principal obstáculo encontrado por indústrias alimentares, ao desenvolver alimentos contendo probióticos, está em como incorporá-los ao alimento, além da escolha da melhor estirpe quanto às suas propriedades de sobrevivência e atividades metabólitas durante a fabricação e armazenamento do alimento (FERNÁNDEZ-PACHECO et al., 2019).

Por apresentarem 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 nesta ampla adaptação são as atividades proteolíticas e lipolíticas e a capacidade de assimilar ou até mesmo fermentar a lactose (ANDRADE et al., 2017; LOPANDICK et al., 2006). A utilização de leveduras como cultura iniciadora na produção de queijos vem sendo amplamente explorada, sobretudo, na fase de maturação de queijos. As leveduras podem contribuir na maturação de queijos em especial por sua eficiência na produção de compostos aromáticos, por suas atividades proteolíticas e lipolíticas, pela interação com outros microrganismos e a capacidade de algumas espécies de consumir a lactose (ATANASSOVA et al., 2016; DOS SANTOS et al., 2017). Price et al. (2014), Padilla et al. (2014) e Zheng et al. (2017) avaliaram o uso de *Debaryomyces hansenii, Yarrowia lipolytica* e *Kluyveromyces lactis* como culturas iniciadoras em queijos e notaram uma grande variedade de compostos aromáticos responsáveis pelo aroma e sabor em queijos, nos queijos fabricados com a adição destas leveduras. Comprova-se, então, que leveduras podem ser utilizadas na produção de queijos e ainda agregar qualidade ao produto.

As leveduras *Torulaspora delbrueckii* B14 e *Kluyveromyces lactis* B10 foram isoladas do processamento do queijo Canastra e apresentaram capacidade de consumir, parcialmente ou por completo, a lactose e de produzir compostos voláteis aromáticos desejáveis (Andrade et al. 2017). Assim sendo, o presente estudo teve como objetivo o uso das leveduras *T. delbrueckii* B14 e *K. lactis* B10 como cultura iniciadoras, na produção de queijos; a sua avaliação quanto à capacidade de sobrevivência à passagem pelo trato gastro intestinal e avaliação de seu potencial tecnológico.

2 REFERENCIAL TEORICO

Queijo

A fabricação de queijos teve seu início há 12 mil anos antes do nascimento de Cristo. Acredita-se que surgiu no "Crescente fértil" entre os rios Tigres e Eufrates, no Iraque, durante a chamada Revolução Agrícola (DE PAULA, DE CARVALHO e FURTADO, 2009). Inicialmente, o queijo era apenas o leite coagulado separado do soro e salgado. Somente na Idade Média que os monges deram início à fabricação de queijos finos, permitindo então que a técnica de sua produçãofosse evoluindo no decorrer do tempo. Ao passar dos anos, o queijo sofreu diversas evoluções até chegar ao que é conhecido atualmente.

Apesar de o Brasil ter sido colonizado por portugueses, um povo que já possuía uma cultura queijeira, a produção de queijos, no Brasil, começou devagar, sendo produzido em escala doméstica e de forma rudimentar. Algumas fazendas de Minas Gerias, que eram situadas às margens do caminho que levava à capital do império, comercializavam seus queijos, no mercado do Rio de Janeiro, os quais apresentavam uma boa aceitação de seus consumidores. Sendo assim, o primeiro queijo fabricado no Brasil foi o Minas que, a princípio, era um queijo para consumo local, porém seu consumo foi se estendendo até chegar a ser um produto altamente consumido nas grandes capitais. A partir do queijo Minas, originaram-se outros tipos de queijos, como o Minas Frescal, Minas Curado e/ou Minas Padrão, queijo do Serro, queijo coalho, entre ouros (Leandro, 1987).

Segundo o Regulamento Técnico Brasileiro de Identidade e Qualidade dos Queijos, regulamentado pela Portaria 146 de 1996, o queijo é o produto fresco ou maturado obtido, a partir da separação parcial do soro do leite ou do leite reconstituído (integral, parcial ou totalmente desnatado), ou de soros lácteos. A coagulação é feita pela ação física do coalho, de enzimas específicas, bactéria específica, ácidos orgânicos, isolados ou combinados, sendo todos esses constituintes de alta qualidade e próprios para o uso alimentar. O queijo pode ser adicionado a substâncias alimentícias, especiarias, condimentos, aditivos especificamente indicados, aromatizantes e corantes. O queijo fresco é o queijo que, logo depois de sua fabricação, está pronto para consumo. O queijo maturado sofre trocas bioquímicas e físicas que são necessárias para as características da grande variedade do queijo. A denominação queijo é exclusivamente

aos produtos em que a base láctea não contém gordura e/ou proteínas de origem não láctea (BRASIL, 1996).

De acordo com Brasil (1996), os queijos podem ser classificados, quanto ao teor de gordura no extrato seco (GES), umidade e ao tratamento térmico realizado após a fermentação.

Em relação ao teor de gordura no extrato seco, os queijos são classificados como:

- ➢ Duplo creme ou extra gordos com, no mínimo, 60 %
- ➢ Gordo, entre 45 a 59,9 %
- ➢ Semigordo, entre 25 a 44,9 %
- ➢ Magro, entre 10 a 24,9 %
- ➢ Desnatado, menor que 10 %

Quanto ao teor de umidade, os queijos são classificados como:

- ➢ Queijos duros (baixa umidade) até 35,9 %
- ➢ Queijos semiduros (média umidade), 36 a 45,9 %
- ➢ Queijos de "massa branda ou macia" (alta umidade), 46 a 54,9 %
- ➢ Queijos de "massa branda ou mole" (muito alta umidade), acima de 55 %

Quando submetidos ou não a tratamento térmico, logo após a fermentação, os queijos de muito alta umidade são classificados em:

- ➢ Queijos de muito alta umidade tratados termicamente.
- ➢ Queijos de muito alta umidade.

Dentre os produtos lácteos, o queijo é o produto mais diversificado, é bioquímica e biologicamente dinâmico. A produção de queijos representa uma série de eventos bioquímicos que levam a produtos com aromas e sabores desejáveis. São produzidos dois tipos de queijos, o fresco e maturado. Queijo fresco é o queijo que, logo após sua fabricação, é consumido, enquanto o queijo maturado é submetido a um período de maturação em condições de temperatura e umidade controladas. Os queijos maturados ainda podem ser subdivididos em duas fases, a fabricação e a maturação. A fabricação é composta pelas fases: adição da cultura starter, coagulação, desidratação (corte do coágulo, cozimento, agitação da massa e pré-prensagem), sinérese, enformagem/modelagem, prensagem e salga. Durante o processo de maturação, há a

produção de diversos compostos voláteis que são responsáveis pela formação do sabor e aroma do queijo. Ainda, na maturação, ocorrem diversas reações bioquímicas, que podem ser divididas em três grupos principais: 1- glicólise da lactose residual e o catabolismo de lactato e citrato; 2- catabolismo dos ácidos graxos livres e lipólise; e 3- catabolismo dos aminoácidos e a proteólise (FOX et al., 2017).

Culturas iniciadoras

Para a fabricação da maioria dos queijos, há a adição de culturas iniciadoras que são compostas por bactérias do ácido lático (BAL) com principal função de produzir ácido lático, a partir da lactose, a fim de auxiliar na coagulação do leite. Essas culturas estão também envolvidas, no processo de maturação dos queijos, pois, no decorrer da maturação, são responsáveis por alterações bioquímicas que auxiliam no desenvolvimento do aroma e sabor característico de cada queijo. As principais espécies envolvidas incluem *Lactococcus lactis*, espécies de *Leuconostoc*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii subsp. lactis*, *Lactobacillus delbrueckii subsp. bulgaricus* e *Lactobacillus helveticus,* contudo nem todas essas bactérias são utilizadas em todas as variedades de queijos (PARENTE, COGAN e POWELL, 2017).

As culturas mesofílicas são aquelas que se desenvolvem melhor a 25 a 30 °C, e as termofílicas a 40 a 45 °C. As culturas mesofílicas contêm cepas acidificantes ou uma mistura de BAL acidificante com fermentadoras de citrato. A BAL mais utilizada, em culturas mesofílicas, é o *Lactococcus lactis*, incluindo as subespécies *L. lactis* ssp. *lactis* e *L. lactis* ssp. *cremoris*. Algumas culturas mesofílicas também podem conter cepas de *Leuconostoc spp.* para a fermentação de citrato. As culturas termofílicas são, em geral, utilizadas, para a produção de queijos duros ou semiduros, utilizando linhagens de *Streptococcus thermophilus* em conjunto com estirpes de *Lactobacillus helveticus* e *Lactobacillus delbrueckii* para o desenvolvimento do sabor (BLAYA, BARZIDEH, LAPOINTE, 2018).

De acordo com seu papel, a cultura iniciadora envolvida, na fabricação de queijos, pode ser dividida em primária ou secundária. A cultura primária é composta basicamente por BAL e está envolvida na produção do ácido lático, no entanto, também, pode produzir compostos voláteis como diacetil que é um importante componente de aroma em queijos. A cultura primária também produz CO_2 por meio de bactérias

heterofermentativas, citrato por bactérias homofermentativas e heterofermentativas queirão contribuir, para a textura do queijo e enzimas proteolíticas, que propiciam o sabor e aroma em queijos maturados (PARENTE, COGAN e POWELL, 2017). As culturas secundárias incluem leveduras, fungos filamentosos e bactérias, sendo os mais comuns *Geotrichum candidum*, *Debaryomyces hansenii*, *Penicillium camemberti*, *Penicillium roqueforti*, *Brevibacterium*, *Corynebacterium* e *Staphylococcus*. O uso das culturas primárias, em conjunto com as secundárias, melhoram as propriedades sensoriais do queijo e os benefícios à saúde do consumidor, tendo em vista que algumas bactérias são probióticas (IRLINGER; HELINCK; JANY, 2017).

A cultura secundária é tão importante quanto a cultura primária, pois pode produzir olhaduras (produção de gases), coloração e desenvolvimento de sabor característico. A cultura secundária é composta por leveduras, fungos filamentosos e bactérias e se desenvolve, principalmente, na superfície do queijo. Essa diversidade de microrganismo depende da qualidade microbiológica do leite, em seu manuseio e tratamento térmico efetuado, das condições de fabricação, manuseio da coalhada, da salga, maturação e da exposição do queijo após sua fabricação (BANJARA; SUHR; HALLEN-ADAMS, 2015).

No início da fabricação de queijos, as BAL são os microrganismos dominantes, porém, no processo de maturação, as leveduras e fungos filamentosos são os microrganismos dominantes da superfície do queijo. Por este motivo, as leveduras são utilizadas, principalmente, na superfície de queijos curados e crescem já nos primeiros estágios da maturação (IRLINGER; HELINCK; JANY, 2017).

As leveduras podem exercer nos queijos funções como interação com diferentes microrganismos de diferentes formas, inibindo ou eliminando os microrganismos causadores de defeitos ou patogênicos, podem vir a inibir culturas iniciadoras, ou contribuir de forma positiva aos processos de maturação e fermentação, apoiando essas mesmas culturas de forma favorável com as características sensoriais do queijo e pela produção de enzimas lipolíticas e proteolíticas (JAKOBSEN e NARVHUS, 1996; BORELLI et al., 2006).

Probiótico

O consumo de probióticos começou juntamente com o de leite e outros alimentos fermentados, porém os efeitos benéficos dos microrganismos foram descobertos somente em 1907 quando Metchikoff sugeriu que a microflora intestinal apresentava efeitos à saúde e a chamou de "auto-intoxicação". Metchikoff ainda sugeriu que o consumo de leites fermentados melhorou esta condição de autointoxicação (KUMAR; VIJAYENDRA; REDDY, 2015).

Probiótico é um termo derivado de uma palavra grega que significa para a vida. Esse termo também é utilizado, para definir microrganismos vivos, que causam benefícios ao seu hospedeiro (PANDEY et al. 2015). Segundo a FAO/WHO, 2002, probióticos são definidos como microrganismos vivos, que, quando ingeridos em quantidades adequadas, conferem beneficios à saúde de seu hospedeiro.

Dentre os benefícios gerados, ao ingerir probióticos, pode-se citar a prevenção de diarreia, ação anti-inflamatória, prevenção à prisão de ventre, entre outras. Também podem contribuir, a fim de melhorar a biodisponibilidade, para a síntese de alguns nutrientes, e alguns probióticos exercem atividade antioxidante (PANDEY et al. 2015). Alguns probióticos não colonizam o intestino, para conceder efeitos benéficos à saúde, esses probióticos, como *Bifidobacterium longum* e *Lb. casei*, agem de forma transitória no intestino a fim de restaurar e manter a homeostase da microbiota intestinal (OHLAND; MACNAUGHTON, 2010).

Para que o probiótico possa exercer com eficácia seus efeitos benéficos à saúde, é necessário que o alimento seja estável, durante toda a sua vida de prateleira, pois o alimento ao qual o probiótico está presente desempenha um papel vital na sobrevivência, estabilidade, fisiologia e na interação dos microrganismos probióticos com outros microrganismos presentes no alimento. Algumas condições de fabricação, como calor, estresse osmótico e danos mecânicos também podem afetar a viabilidade das células probióticas no alimento. Um dos principais critérios na avaliação, para o sucesso ou não do produto no mercado, é a caracterização sensorial (KUMAR; VIJAYENDRA; REDDY, 2015).

Os probióticos são mais consumidos, em forma de alimentos fermentados, sendo os produtos lácteos, como leites fermentados, iogurtes e queijos os principais portadores de probióticos consumidos. Atualmente o estudo de alimentos adicionados de probióticos vem sendo alvo da população científica, e a maioria dos estudos são concentrados em alimentos lácteos (AMORIM; PICCOLI; DUARTE, 2018).

A maioria dos microrganismos probióticos são bactérias, sendo as mais utilizadas industrialmente *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Bifidobacterium* e certas estirpes de *Lactobacillus casei*, grupo *Lactobacillus acidophilus* e *Bacillus coagulans* (PANDEY et al., 2015, GIBSON et al., 2017). Dentre as leveduras, somente a levedura *Saccharomyces cerevisiae* var. *boulardii* é considerada probiótica, porém diversos autores sugerem a avaliação de outras especies de leveduras quanto à sua capacidade probiótica. Inicialmente, antes de realizar os testes, para definir o potencial probiótico do microrganismo, deve ser avaliado se o microrganismo possui capacidade de sobreviver pela passagem pelo trato gastrointestinal, pela tolerância a baixo pH, sais biliares e enzimas gástricas (PAPADIMITRIOU et al., 2015; FADDA et al., 2017).

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SEGUNDA PARTE – ARTIGO

ARTIGO 1 - Survival of *Kluyveromyces lactis* and *Torulaspora delbrueckii* to simulated gastrointestinal conditions and their use as single and mixed inoculum for cheese production

Survival of *Kluyveromyces lactis* and *Torulaspora delbrueckii* to simulated gastrointestinal conditions and their use as single and mixed inoculum for cheese production

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Abstract

The demand for new probiotic products has shown recent increases alongside a growing interest in studying starter cultures of cheeses. This study thus aims to evaluate the ability to survive under simulated gastrointestinal conditions and impact of *Torulaspora delbrueckii* B14 and *Kluyveromyces lactis* B10 as single and mixed inocula for cheese production. These two yeast strains were subjected to simulated gastrointestinal tracts and tested for self-aggregation, hydrophobicity, pathogen inhibition, antibiotic resistance, and β-galactosidase production. The yeast strains were also assessed for their ability to survive in different NaCl concentrations (2.5%, 5%, and 10% w/v), multiple temperatures (4 ºC and 40 ºC), and used as single and mixed starter cultures for cheese production. Yeasts population levels were monitored by YPD plating and MALDI-TOF and metabolites were analyzed by HPLC and GC-MS over the course of the 21 days cheese maturation process. *T. delbrueckii* B14 and *K. lactis* B10 both showed >80% viability after the passage through the simulated gastrointestinal tract, had self-aggregation rates >90%, and displayed β-galactosidase activities of 0.35 U/g and 0.53 U/g, respectively. Both yeasts survived at 2.5%, 5%, and 10% NaCl for 21 days and showed growth at 4 ºC. In cheese, the single inoculum of K. *lactis* B10 and mixed inoculum showed the highest levels of lactose consumption. HS-SPME GC-MS analysis of cheese samples allowed the identification of 38 volatile compounds. The highest concentrations of most of these compounds were observed after 21 days of maturation for the cheese produced with mixed inoculum. The most abundant acids detected were hexanoic and decanoic acid; the most abundant alcohols were 2,3-butanediol, 2-phenylethanol and isoamyl alcohol, and the most prevalent ester compounds were isoamyl acetate and phenethyl acetate. Our results therefore show that *T. delbrueckii* B14 and *K. lactis* B10 are interesting yeasts for further studies in the context of probiotics and positively impact the composition of desirable volatile compounds in cheeses, particularly when used as mixed inoculum.

Keywords: starter culture; yeasts; volatile compounds; HPLC; GC-MS.

1 INTRODUCTION

Yeasts are often present during the cheese production process and can be found in equipment, brine, and starter cultures (Andrade, Melo, Genisheva, Schwan, & Duarte, 2017; Gardini et al., 2006). Recent studies have reported the presence of yeasts in different types of cheese. Species such as *Yarrowia lipolytica* (Ceugniez et al., 2017a), *Torulaspora delbrueckii* (Andrade et al., 2017) and the genus *Kluyveromyes* (Andrade et al., 2017; Dos Santos, Benito, Córdoba, Alvarenga, & Herrera, 2017) are frequently associated with cheese maturation. Yeast has also been isolated from natural cheese starts as reported by Andrade et al. (2017) and Coloretti et al. (2017). These microorganisms are well adapted to growing in cheese production conditions owing to their tolerance of low pH, high salt concentrations, low temperatures, and low water activity (Ferreira & Viljoen, 2003). In addition, yeasts are able to assimilate lactose, enabling their growth in cheese.

Because of their proteolytic activity, lipolytic activity, and aromatic compound production, yeast may play an important role during cheese maturation (Atanassova et al., 2016; Gardini et al., 2006; Padilla, Belloch, López-Díez, Flores, & Manzanares, 2014). In fact, the use of yeast in cheese production has been extensively studied in the recent years, although not much exploration has been done for industrial production. For example, multiple studies have evaluated the performance of *Debaryomyces hansenii, Yarrowia lipolytica*, and *Kluyveromyces lactis* in cheese production and *K. lactis* appears to impact aromatic compound production (Arfi et al., 2004; Martin,

Berger, & Spinnler, 2002; Padilla et al. (2014); Price et al., 2014; Zheng et al., 2017). The use of yeast in cheeses has also been considered owing to their ability to inhibit growth of some microorganisms known to cause cheese spoilage such as *Pseudomonas* and *Clostridium*.

Yeast appear to have some probiotic potential, as demonstrated for *Saccharomyces cerevisiae*, *Saccharomyces unisporus, Galactomyces geotrichum,* and *K. lactis* (Binetti, Carrasco, Reinheimer, & Suárez, 2013; Chen et al., 2010; Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014; Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017). The probiotic potential of *K. lactis* and *K. marxianus* was demonstrated by Ceugniez, Coucheney, Jacques, Daube, Delcenserie, and Drider, (2017b). These authors evaluated the anti-*Salmonella* activity of both strains, and *K. marxianus* S-2-05 showed inhibitory effect on the bacteria, reinforcing its probiotic potential. Recently, Cho et al. (2018) reported that strains of *K. marxianus* were more efficient in adhering to intestinal cells than *Lactobacillus acidophilus,* a bacteria widely recognized as probiotic.

Canastra cheese is one of the most famous cheeses in Brazil and is produced from raw milk in the Serra da Canastra region, located in the southwest of Minas Gerais State. Besides commercial rennet, an endogenous starter called "pingo" is used to produce Canastra cheese and is obtained from cheese whey produced the previous day. The pingo microbiota is composed mainly of lactic acid bacteria and yeasts (Lima, Lima, Cerqueira, Ferreira, & Rosa, 2009). One hour after rennet and pingo additions, the cheese is cut and transferred to plastic molds for cheese shaping and the whey removed by hand pressure. The salting process is performed by adding NaCl on one side of the cheese then, 6 h later, adding NaCl to the other side of the cheese. After salting, the cheese is matured on wooden shelves at room temperature, which can vary

from 3 to 30 days for Canastra cheese (Borelli, Ferreira, Lacerda, Franco, & Rosa, 2006; Lima et al., 2009). Andrade et al. (2017) isolated the yeasts *K. lactis* B10 and *T. delbrueckii* B14 from Canastra cheese production process. These strains were also found at high levels in the mature cheese ingested by consumers. In this work we aimed to assess the survival of *T. delbrueckii* B14 and *K. lactis* B10 under simulated gastrointestinal conditions, evaluate their survival of different temperatures and NaCl concentrations, and determine their impact as single or mixed inoculum in the aromatic volatile compounds in cheese over 21 days of maturation.

2 Materials and Methods

2.1 Chemicals

Sodium chloride, KCl, Na_2HPO_4, KH_2PO_4, $CaCl_2$, $NaHCO_3$, $MgSO_4 \cdot 7H_2O$ and Na_2CO_3 were purchased from Labsynth Brazil. Pancreatin from porcine pancreas, ox-bile, n-hexadecane, ampicillin, chloramphenicol, erythromycin, G penicillin, streptomycin, tetracycline, nystatin, ONPG, YNB and sulfuric acid were purchased from Sigma Aldrich.

2.2 Microorganism and inoculum preparation

Torulaspora delbrueckii B14 and *K. lactis* B10 were previously isolated from Canastra cheese production by Andrade et al. (2017). Cultures were stored in 20% glycerol at -80 ℃ and reactivation performed in 1 mL of YPD (1% yeast extract; 2% peptone; 2% glucose) at 30 ℃ for 24 h. Cell growth was triggered by diluting cultures 1:10 in increasing volumes of media until the desired amount of cells was obtained for each experiment (Andrade et al., 2017).

2.3 Evaluation of potential for use in cheese production

Cultures were centrifuged for 10 min at 4 ºC, 14,224 g then washed twice with PBS (Phosphate-Buffered Saline; 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4). The obtained biomass was used as inoculum for tests described below. All experiments were performed in triplicate.

2.3.1 Resistance to simulated gastrointestinal conditions

In this test, cells were exposed to conditions mimicking those present during passage through the stomach and intestines. Yeast inoculum obtained as previously described (item 2.2) was first inoculated into 10 mL of simulated gastric juice (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$, 0.3% pepsin, and pH 3.0) to a final population of 10^8 cells/mL and shaken at 37 ºC, 150 rpm. After 90 min of incubation, 17.5 mL of synthetic duodenum juice (6.4 g/L de $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.1% pancreatin, 10% ox-bile, pH adjusted to 7.4 with 5 M HCl) was added to simulate the passage through the upper intestinal tract and samples shaken at 37 ºC, 150 rpm for more 180 min. Yeast survival rate was then assessed by growth on YPD plates incubated at 30 ºC for 48 h (Fadda et al., 2017).

2.3.2 Self-aggregation

Self-aggregation tests were performed according to Fadda et al. (2017) with modifications. The inoculum previously obtained (item 2.2) was resuspended in 3 mL of PBS buffer to a final concentration of 10^8 cells/mL, vortexed for 30 s, then transferred to a glass cuvette to measure the optical density (OD) for the 0 h timepoint. The solution was then maintained in the cuvette without disturbance for 24 h at 37 ºC and OD readings taken after 2 h, 4 h, and 24 h. The percentage of self-aggregation was calculated by the equation:

Self-aggregation %= (At/A0)*100

where At represents the absorbance at either 2 h, 4 h, or 24 h and A0 is the absorbance at the 0 h (T0).

2.3.3 Hydrophobicity

To measure hydrophobicity, 3 mL of yeast cells suspended at 10^8 cells/mL in PBS was added of 1 mL of n-hexadecane and vortexed for 120 s. The solution was then incubated at 37 ºC for 1 h to allow for phase separation. After incubation the aqueous phase was carefully removed and the OD of the remaining fraction measured at 560 nm. Hydrophobicity was then calculated according to the following equation:

Hydrophobicity = [(OD0 - OD) / OD0]*100

where OD0 corresponds to the optical density before extraction and OD refers to the optical density after extraction with n-hexadecane.

2.3.4 Antagonism against pathogenic bacteria

The ability of yeast species to inhibit growth of *Salmonella enterica* serovar Enteritidis ATCC 5190, *Escherichia coli* ATCC 055, and *Listeria monocytogenes* ATCC 11778 was evaluated using three different methods as described in Ceugniez, Drider, Jacques and Coucheney (2015). First, using the late method, 100 μL of yeast culture at 10^8 cells/mL was plated on a YPD plate. After growth at 28 ºC at 48 h, colonies were removed with a platinum loop, and plates inoculated with 100 μL of pathogen culture at 10^4 cells/mL then incubated at 37 ºC for 48 h. In the supernatant method, 100 μL of pathogen culture (10^4 cells/mL) was spread on a Tryptic Soy Agar (TSA) plate. 7 wells of 5 mm diammeter were then made on each plate and filled with approximately 20 μL of 0.22 μm filtered yeast growth medium. To allow the growth

media, plates were kept at 4 ºC for 2 h then incubated at 37 ºC for 48 h. Finally, the gas production method was performed using two compartments plates by inoculating the yeast culture in one compartment and the pathogen in the other compartment. Plates were then incubated at 37 ºC for 48 h. To obtain results for each of these protocols, the presence or absence of inhibition halos was evaluated.

2.3.5 Antibiotic resistance

Antibiotic resistance tests were carried out using ampicillin (10 and 25 μg/mL), chloramphenicol (30 and 60 μg/mL), erythromycin (5, 15 and 78 μg/mL), G penicillin (10 μg/mL), streptomycin (25 and 100 μg/mL), and tetracycline (30 and 80 μg/mL) according to Perricone, Corbo and Sinigaglia (2014) and Syal and Vohra (2013). Yeast cells were spread on YPD agar with a sterile swab and 10 μL of each antibiotic deposited on the plate and antibiotic sensitivity evaluated based on the formation of inhibition halos around the antibiotic drop (Todorov et al., 2008). Yeast sensitivity to nystatin was evaluated using 4 mL of nystatin (100,000 IU) per liter of YPD. Yeast cultures were spread on the YPD plates containing nystatin with a sterile swab and plates incubated at 30 ºC for 48 h.

2.3.6 Production of β-galactosidase

The evaluation of β-galactosidase activity was performed by inoculating 10^7 yeast cells/mL in 10 mL of enzyme production medium (3% lactose, 0.7% yeast extract, 0.3% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4·7H_2O$, and 0.3% K_2HPO_4) (Song, Liu, Xu and Chi, 2010), with incubation at 30ºC, 200 rpm for 48 h. Cells were then centrifuged at 4 ºC and 14,224 g for 10 min and the supernatant discarded. The recovered cells were permeabilized to release the enzyme by resuspending 500 mg of biomass in 5 mL of isoamyl alcohol and disrupted with glass beads (1 mm diameter) by vortexing for 5 min. Enzymatic activity was then evaluated according to Cardoso et al. (2015). Two hundred

microliters of yeast cells extract were added to 800 μL of 2.5 mg/mL ortho-nitrophenyl beta-D-galactopyranoside (ONPG) in 0.1 M phosphate buffer and incubated at 37 ℃ for 15 min. The reaction was stopped by the addition of 200 μL of Na_2CO_3 1 mM and the absorbance at 420 nm measured. Enzyme activity was calculated according to the Food Chemical Codex (1993) based on an ONPG extinction coefficient of 4.6 mM.

2.4 Yeast survival at different temperatures and NaCl concentrations

Yeast survival in varying NaCl concentrations and temperatures was evaluated using protocols from Binetti et al. (2013) and Atanassova et al. (2016) with some modifications. To test effects of varying temperatures, yeast was grown in YPD broth and incubation at either 4 ℃ and 40 ℃. To test effects of different NaCl concentrations, yeast was grown in YNB medium supplemented with 0.5% glucose and NaCl concentration of 2.5, 5 or 10% w/v. For both tests, yeast viability was checked every 7 days for 21 days by plating 100 μL on YPD media, incubation at 30 ℃ and colony counts.

2.5 Cheese production

The milk used for cheese production was obtained from a farm in Lavras – MG/Brazil. contained 4.1% of fat, 18 ℃D acidity, pH 6.6, and a density of 1030 at 15 ℃.

The cheeses were produced using pasteurized milk (65 ℃ for 30 min). Fifteen liters of cooled (38 ℃) pasteurized milk were transferred to stainless-steel tanks and a single inoculum of each yeast or mixed inoculum (1:1) with both yeast species at 10^5 cells/mL was inoculated into the milk immediately after cooling. Milk coagulation was triggered by the addition of commercial rennet (HA-LA® Chr. Hansen Brazil) to inoculated milk at a concentration of 1 mL/L (75 IMCU/mL). After 30 min, the obtained solids were cut with a stainless-steel curd cutter (curd harp), allows to rest

undisturbed for 2 min, then stirred for 35 min. The resulting whey was then removed and the cheese mass placed into molds (120 mm diameter x 110 mm height). Dry salting was carried out by distributing 50 g of NaCl on the upper surface of each cheese. After 150 min, excess salt was removed and added to the non-salted side of the cheese. After 150 min, the excess salt was again removed and the cheeses removed from molds then stored in a cold room at 10 ºC and 85% humidity for 21 days of maturation.

2.6 Yeast population monitoring and MALDI-TOF MS analysis

Samples of cheese were collected every 7 days by introducing a sterile stainless-steel probe from the surface to the center of the cheese. These samples (2.5 g) were diluted in 5 mL of 0.1% sterile peptone, homogenized in a vortex for 1 min, and aliquots of 100 µL plated on YPD plates containing 0.01% chloramphenicol then incubated at 30 ºC for 24 h. Colonies were counted, then five colonies from each plate were randomly collected and analyzed by MALDI-TOF MS. Protein extraction and MALDI-TOF MS analysis for yeast species identification were performed according to Amorim, Schwan, and Duarte (2016) and Usbeck, Kern, Vogel, and Behr (2013). Briefly, 900 µL of cell suspension was transferred to a 1.5 mL tube and centrifuged at 25,200 g and 4 ºC for 2 min. The supernatant was removed and the cell pellet mixed with 300 µL ultrapure water followed by the addition of 900 µL absolute ethanol. After centrifugation at 25,200 g and 4 ºC for 2 min, the ethanol was decanted for 5 min and the spin repeated. The supernatant was discarded, the pellet, air-dried and then resuspended in 50 µL 70% formic acid. An equivalent volume of acetonitrile was then added to the suspension, which was centrifuged at 25,200 g, 4 ºC for 2 min. One microliter of the supernatant was spotted onto a MALDI target and dried at room temperature. The target spots were covered with 1 µL of α-cyano-4-hydroxy-cinnamic acid (10 mg/mL). Analyses were performed using a Microflex LT spectrometer

(Bruker) and FlexControl software (Version 3.0). Yeast identity confirmation was performed by comparing the protein profiles with those previously obtained by Andrade et al. (2017) for the same strains used in this work and also using the Bruker Biotyper Library. Data obtained from the MALDI-TOF analysis were used in a cluster analysis as described by Amorim et al. (2016). Briefly, the raw spectra were processed using the mMass software version 5.5 (Niedermeyer & Strohalm, 2012), and for each sample group an average spectrum was obtained. The SPECLUST, available at http:// http://co.bmc.lu.se/speclust/common.pl. (Alm et al., 2006) was used to align the peaks and generate a consensus peak list. This consensus peak list was used for the cluster analysis.

2.7 Analysis of sugars and acids

Samples for sugar and acid analyses were collected at the same time intervals as yeast count samples. Five hundred milligrams of cheese were added to 2.5 mL of mobile phase, vortexed for 5 min, then centrifuged twice at 4 ºC, 11,200 g for 10 min and filtered with 0.22 µm pore filters. Chromatographic analysis was performed on a Shimadzu (Shimadzu Corp., Japan) chromatograph equipped with a refractive index detector (RID-20A) and DAD detector (PDA). Compound separation was performed on a Supelcogel 8H column (7.8 mm x 30 cm) operated at 30 ºC with a 5 mM sulfuric acid mobile phase and a flow rate of 0.5 mL/min. Compound identification was performed by comparing retention times of experimental sample peaks with the retention times of reference standards injected under the same conditions. Quantification was performed using external calibration curves (Andrade et al., 2017; Duarte et al., 2010) and all analyses were performed in duplicate.

2.8 Analysis of volatile compounds by HS-SMPE and GC-MS

Volatile compound extraction was carried out using 3 g of cheese (Hayaloglu, Brechany, Deegan, & McSweeney, 2013) mixed with 4-nonanol as an internal standard to a final concentration of 125 μg/Kg in 15 mL vials. Vials were then incubated at 65 ºC for a total of 35 min. During the first 10 min of incubation, volatiles were allowed to be collected in the headspace and then extracted during the following 25 min using a 50/30 μm DVB/CAR/PDMS fiber. The fiber was kept for 5 min in the injector at 230 ºC for desorption of volatiles then analyses performed on a GC-MS QP2010SE (Shimadzu) according to Andrade et al. (2017). The compounds were identified using the NIST 2011 library and identities were confirmed using the linear retention index (LRI) calculated from the injection of a c8-c40 alkane series. Concentrations were expressed as 4-nonanol equivalents.

2.9 Statistical analysis

The Sisvar 5.6 software (Lavras-MG) was used for ANOVA and Scott-Knott test. Cluster analysis was performed using Euclidean distance and Unweighted Pair-Group Average (UPGA) in XLstat 2014.5 software (Addinsoft's, New York, NY).

3. Results and discussion

3.1 Passage through simulated gastrointestinal conditions

According to Pennacchia, Blaiotta, Pepe, and Villani (2008), a survival rate ≥ 70% is required to define the probiotic potential of a yeast. Therefore, we evaluated survival of *K. lactis* B10 and *T. delbrueckii* B14 to simulated gastrointestinal conditions. After passage through simulated gastric juice for 90 min, *K. lactis* B10 and *T. delbrueckii* B14 showed survival rates of 90.02% and 90.45%, respectively (Figure 1). Upon passage through the whole simulated digestive tract over the course of 270 min, *K. lactis* B10 and *T. delbrueckii* B14 showed survival rates of 81.5% and 84.67%,

respectively (Figure 1). The resistance to pancreatic secretions is a prerequisite for the microorganism to be considered as probiotic since these secretions have several enzymatic properties that can influence the viability of these microorganisms, preventing their survival and colonization of the colon mucosa. Due to the instability of the secretions and the difficulty of obtaining them, most studies use artificial pancreatic secretions (Marteau, Minekus, Havenaar and Huis, 1997). Therefore, commercial pancreatin (Creon®) was added to the synthetic duodenal juice. Similar to our results, Binetti et al. (2013) and Živković et al. (2015) have demonstrated the probiotic potential of *K. lactis* strains, with Binetti et al. (2013) demonstrating one *K. lactis* strain lost 81% of viability, while other 4.5% after exposure to simulated gastrointestinal conditions. *T. delbrueckii* isolated from Feta cheese (Psomas, Andrighetto, Litopoulou-Tzanetaki, Lombardi and Tzanetakis, 2009) survived in the conditions to which it was exposed being considered as possible probiotics. It is noteworthy that the survival rates presented by the *K. lactis* B10 and *T. delbrueckii* B14 under the simulated gastric conditions may result from the adaptation to the conditions found in the environment (Canastra cheese production process) from which they were isolated. Mainly in "Pingo" and cheese,

yeasts coexist with lactic acid bacteria that make the substrate more acidic.
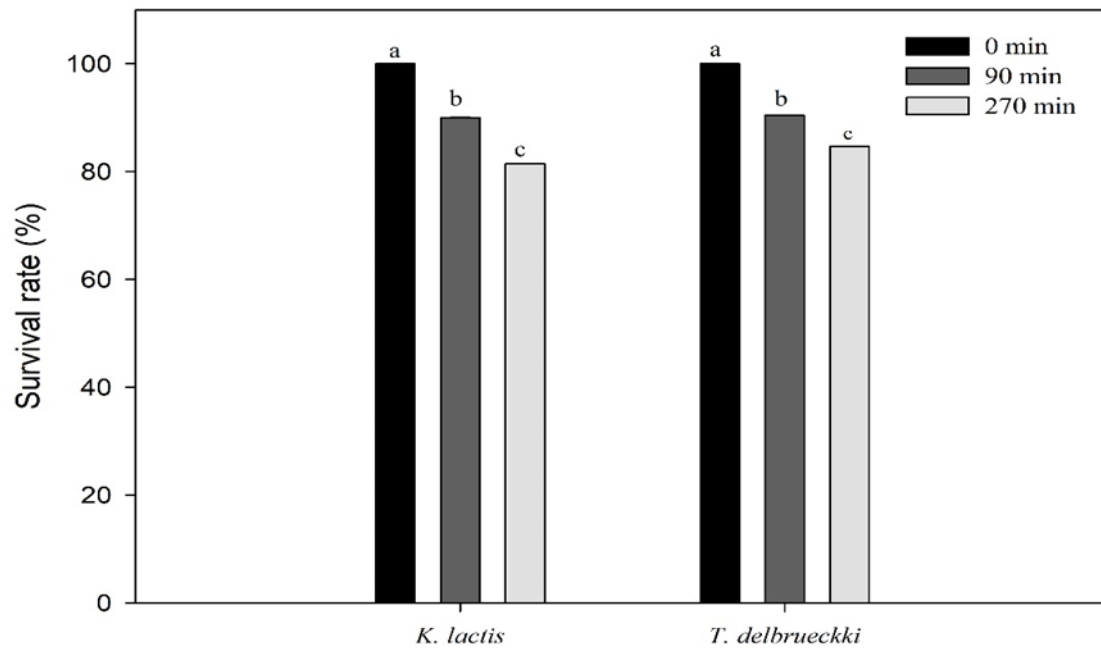
Figure 1. Different letters indicate the existence of statistical difference between different times by the Scott-Knott test for $p \leq 0.05$.

3.2 Self-aggregation and hydrophobicity

Hydrophobicity and self-aggregation play important roles in adhesion and proliferation of microorganisms in intestinal epithelial cells, with high rates of hydrophobicity indicating a strong ability of a microorganism to adhere to intestinal mucosa. The adhesion of probiotic microorganisms to the intestinal wall prevent their elimination through peristaltic movements and prevents pathogenic or undesirable microorganisms from adhering to intestinal mucosa (Chen et al., 2010; Kos et al., 2003; Kumura, Tanoue, Tsukahara, Tanaka, & Shimazaki, 2004). *K. lactis* B10 and *T. delbrueckii* B14 showed statistically different self-aggregation rates after both the 2 h and 4 h of incubation. After 2h of incubation, self-aggregation rates were 54.48% and 60.67% for *K. lactis* B10 and *T. delbrueckii* B14, respectively, while these rates increased to 84.26% and 81.39%, respectively, after 4 h of incubation (Figure 2). The self-aggregation rates after 4 h suggest that these yeast species will be effective in

adhering to the intestinal mucosa upon ingestion. In contrast, both yeasts showed similar self-aggregation rate after 24 h of incubation (Figure 2). Previous studies examining the same yeast species show mixed support for our observations on self-aggregation. Similar to our study, Gil-Rodríguez, Carrascosa, and Requena (2015) reported that a *T. delbrueckii* strain showed high (> 85%) aggregation capacity at 2 h and 4 h of incubation. However, Živković et al. (2015) found that *T. delbrueckii* strains from traditional cheeses of Serbia and Croatia had self-aggregation rates of only 40%, thus showing no potential for adhesion. Considering our results and those reported in the literature, there may be a strain-specific dependence of self-aggregation in the case of *T. delbrueckii*. The self-aggregation parameter is reported with large variations of values, depend on, for example, the yeast species. In the work of Fernández-Pacheco, Arévalo-Villena, Rosa and Pérez, (2018), self-aggregation values ranged from 3.85% to 64.43%, respectively for *Ogataea polymorpha* and *Hanseniaspora osmophila*.

K. lactis B10 and *T. delbrueckii* B14 showed hydrophobicity rates of $55.6\pm2.16\%$ and $90.5\pm4.39\%$, respectively. Inferences about the adhesion ability to intestinal mucosa have been made based on self-aggregation and hydrophobicity from *in vitro* tests. Fadda et al. (2017) and Ceugniez et al. (2017), showed that *Kluyveromyces* strain presented probiotic potential, evaluating, among others, self-aggregation and hydrophobicity. The values found for *K. lactis* B10 and *T. delbrueckii* B14 were higher than that reported by Helmya, Soliman, Abdel-Ghany, and Ganash, (2019), whose highest hydrophobicity was 46.18% for *Pichia kudriavzevii* using chloroform as solvent. However, Zeng, Fan, He, Duan, and Xia (2019) reported hydrophobicity ranging from 81% to 87% using hexadecane as solvent.
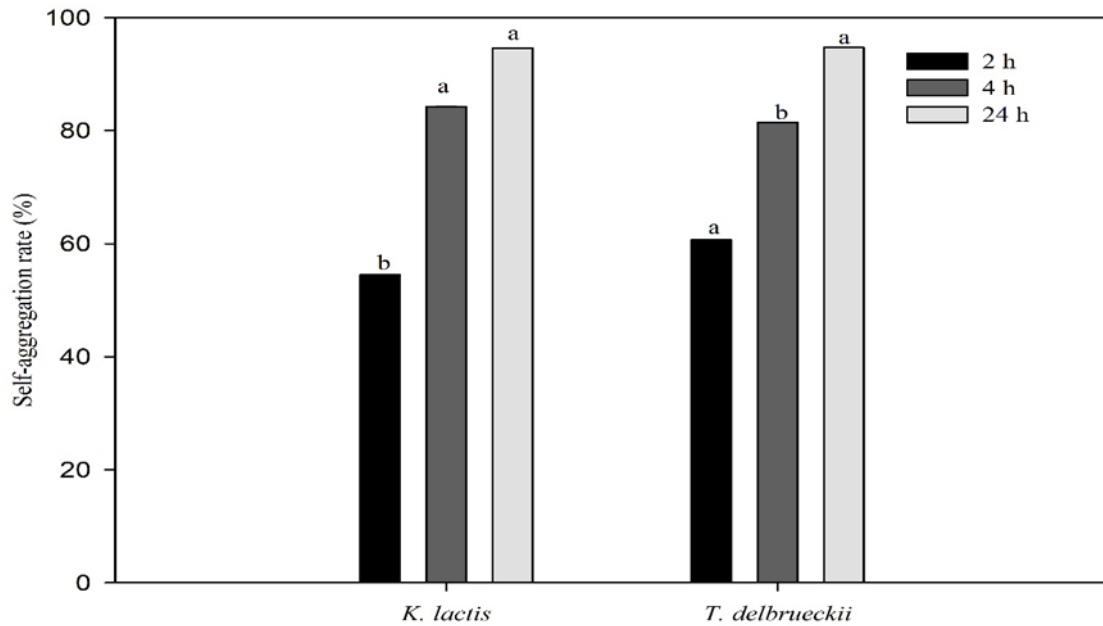
Figure 2. Different letters indicate the existence of statistical difference between different times by the Scott-Knott test for $p \leq 0.05$.

3.3 Antagonism against pathogenic bacteria and antibiotic resistance

K. lactis B10 and *T. delbrueckii* B14 were not able to inhibit the growth of *Salmonella enterica* serovar Enteritidis ATCC 5190, *Escherichia coli* ATCC 055, and *Listeria monocytogenes* ATCC 11778 using the three methods reported above (data not shown). However, it is important to remember that the results are not a definitive measure of the ability of these yeast species to exert antagonistic effects against pathogenic bacteria. Such antagonistic behavior can often rely on particular interactions between a yeast strain and a particular strain of bacteria, thus for *K. lactis* B10 and *T. delbrueckii* B14 further tests are required to fully evaluate the antibacterial properties of these yeast strains. The work of Ceugniez et al. (2017b) is an example of this possible particularity between strains and the inhibitory effect. *Kluyveromyces marxianus* S-2-05 and K. lactis S-3-05 strains isolated from Tomie d'Orchies were evaluated for inhibition of *S. enterica* subsp. *enterica* serovar Enteritidis SR071 and *S. enterica* subsp. *enterica*

serovar Paratyphi B SR425. In this case, the authors demonstrated, via gene expression, the existence of bacteria inhibition by yeast.

As expected, *K. lactis* B10 and *T. delbrueckii* B14 were resistant to ampicillin, chloramphenicol, Erythromycin, G penicillin, Streptomycin, tetracycline and sensitive to nystatin. While probiotic bacteria are not able to resist or tolerate the tested antibiotics, yeasts have resistance to them and can be used in patients undergoing antibiotic treatment (Amorin et al. 2018).

3.4 Production of β-galactosidase

Certain strains of *K. lactis* have been widely studied as a preferred source of β-galactosidase (Song et al., 2010). *T. delbrueckii* is not commonly noted as a β-galactosidase producer, however, some studies such as Borelli et al. (2006) have shown that certain *T. delbrueckii* strains demonstrate efficiency in fermenting lactose, indicating the presence of β-galactosidase activity. The β-galactosidase activities of *K. lactis* B10 and *T. delbrueckii* B14 measured in this study were 0.53±0.02 U/g and 0.35±0.01 U/g, respectively. These values are lower than those reported by Song et al. (2010). However, most studies on β-galactosidase production from *Kluyveromyces* demonstrate the need to optimize process parameters such as cultivation conditions, medium composition, temperature, enzyme extraction, etc. Therefore, in the case of the strains studied here, further tests to optimize enzyme production may result in activities greater than those found in this work.

The ability of some microorganisms to degrade lactose is important in view of the benefits to lactose intolerant people. Indirectly, via the production of the enzyme and its application in food; or directly, via the use of the microorganism itself.

3.5 Yeast survival at varying salt levels and temperatures

NaCl concentrations of 2.5%, 5%, and 10% were chosen based on NaCl concentrations commonly used in cheese salting processes like dry salting, salting the mass directly, and salting using brines (Binetti et al., 2013). At NaCl concentrations of 2.5% and 5%, *K. lactis* B10 populations were statistically indistinguishable after 7 d of growth (Figure 4). Similarly, there was no significant difference in *T. delbrueckii* B14 population levels at 2.5% of NaCl after 7 d and 14 d of growth while the *T. delbrueckii* populations decreased significantly to 10^4 CFU/mL on the 14th day in 5% NaCl (Figure 3). For both species, after 21 d of growth in 2.5% and 5% NaCl, the population levels were approximately 10^4 CFU/mL (Figure 3 and Figure 4). In addition, growth of either species in 10% NaCl did not yield appreciably different population levels compared to other NaCl concentrations examined (Figure 3 and Figure 4).

In terms of temperature sensitivity, only *K. lactis* B10 was able to survive for 7 d when cultured at 40 ºC, however, both strains were able to survive incubation at 4 ºC for at least 21 d (Figure 3 and Figure 4).

Thus, based on NaCl and temperature sensitivity results, both *K. lactis* B10 and *T. delbrueckii* B14 are likely to survive during the maturation period of cheeses like Canastra cheese. This is probably related to the fact that both strains were isolated from Canastra cheese and are already adapted to an environment similar to those tested.
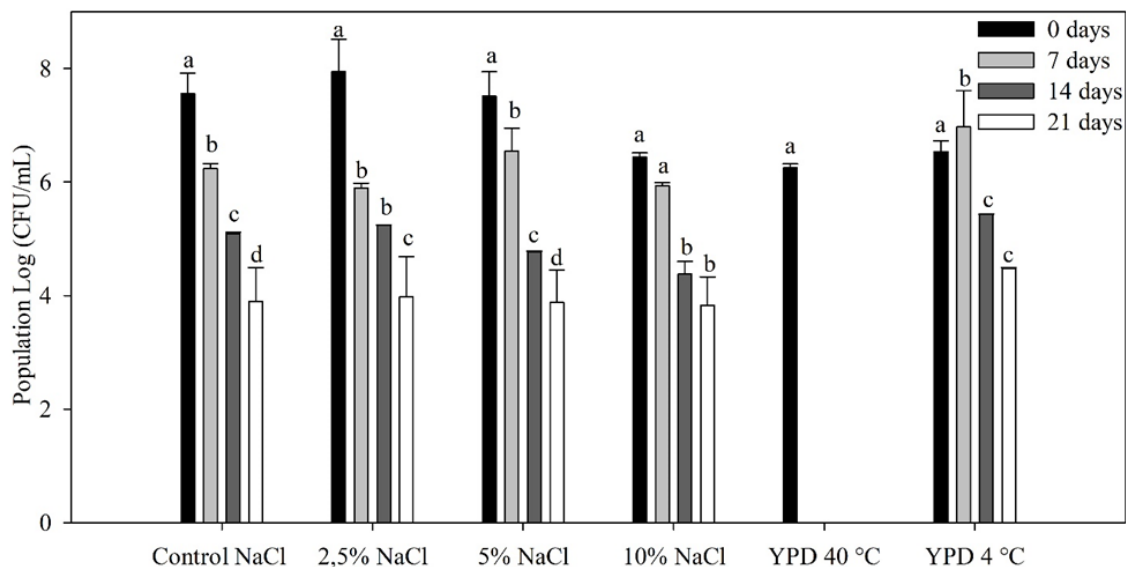
Figure 3. Different letters indicate the existence of statistical difference between different times by Scott-Knott test for $p \leq 0.05$.
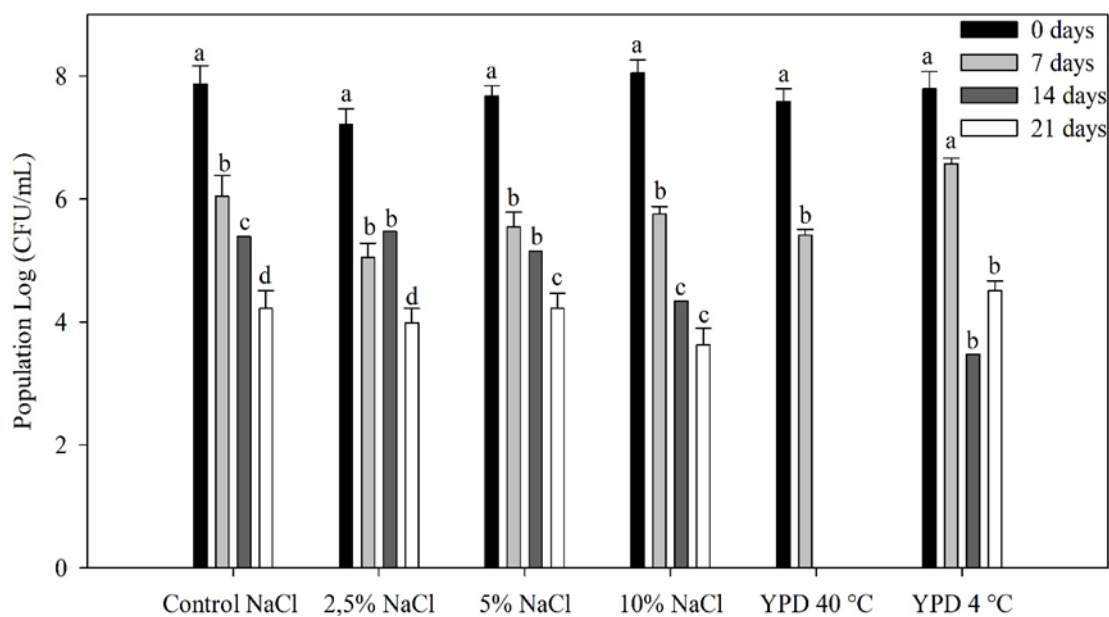
Figure 4. Different letters indicate the existence of statistical difference between different times by Scott-Knott test for $p \leq 0.05$

3.6 Yeast populations during the cheese maturation process

To test cheeses produced using *K. lactis* B10 and *T. delbrueckii* B14. milk was inoculated using either single or mixed inocula at populations of approximately 10^5 cells/mL. In cheese produced with *K. lactis* B10, yeast populations remained stable at 7 d of growth, increased to 3.32×10^6 CFU/mL at 14 d, and grew to 1.2×10^8 CFU/mL at 21

d. Thus, yeast populations at 14 d and 21 d were significantly higher from that at 7 d (Figure 5). After 21 d, the *T. delbrueckii* B14 population in cheese was approximately 10^7 CFU/mL (Figure 5), although this was not statistically different from the population found on the 14th day. The cheese inoculated with the mixed inoculum also exhibited a gradual increase in the yeast population with counts of approximately 10^6, 10^7, and 10^8 CFU/mL on days 7, 14 and 21, respectively (Figure 5). Overall, cheese inoculated with *K. lactis* B10 only and with mixed inoculum showed the significantly highest populations (compared to *T. delbrueckii*) at 21 d of maturation (Figure 5). This may be due to the fact that *K. lactis* is able to consume lactose more efficiently than *T. delbrueckii*, which may be associated with higher β-galactosidase activity as described previously. Thus, in terms of exploring the probiotic character of our chosen yeast species, *K. lactis* B10 and *T. delbrueckii* B14 follow expectations set for probiotics.
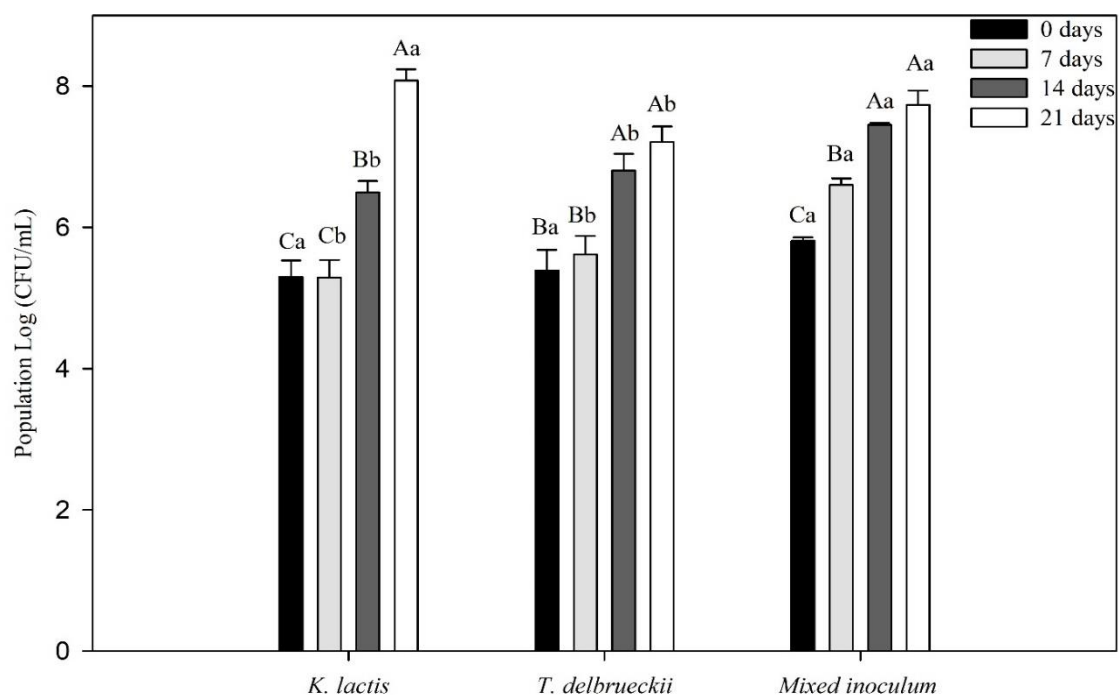
Figure 5. Different lower-case letters indicate the existence of statistical difference ($p \leq 0.05$) between different inocula. Different capital letters indicate the existence of statistical difference ($p \leq 0.05$) between populations for the same inoculum in different times.

From the MALDI-TOF MS analyses of the randomly collected colonies, for cases of single inocula, the identified yeast strains corresponded to those each cheese

was initially inoculated with. In the case of mixed inoculum, it was not possible to accurately determine the proportion of each yeast strain in the total population via YPD plating. The cluster analysis in Figure 6 demonstrates the distinct grouping of the two yeast species re-isolated over 21 days of cheese maturation process. The identification based on Bruker Biotyper library resulted in scores higher than 2.1, which according to the manufacturer, allows identification to the species level.
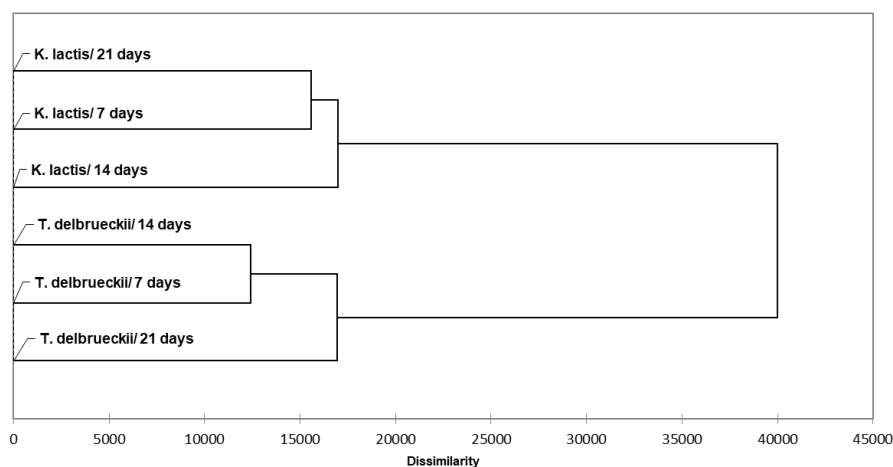
Figure 6. Dendrogram of protein profile of yeasts over 21 days of cheese maturation.

3.7 Analysis of sugars and acids

The cheese inoculated with the *K. lactis* B10 showed lower residual amounts of lactose compared to cheese produced with *T. delbrueckii* B14 (Table 1). As reported above, *K. lactis* B10 is more efficient in producing β-galactosidase, which is associated with the lactose consumption. *K. lactis* B10 was able to consume 76.3% of the lactose in the first 7 d of maturation and 96.7% of the lactose after 21 d of cheese maturation, resulting in 0.88 g of lactose per Kg of cheese (Table 1). The *T. delbrueckii* B14 lactose consumption was slower than that of *K. lactis* B10, resulting in cheese with 8.17 g/kg after 14 days of maturation (Table 1). On the 21st day of maturation, lactose content in the cheese with *T. delbrueckii* B14 was 5.21 g/Kg (Table 1). It was interesting to note

that the mixed inoculum presented intermediate results for lactose consumption throughout the maturation process. After 7 days of maturation, lactose consumption was 43.4% and continued to increase with longer maturation, reaching 3.16 g/Kg after 14 d (Table 1), corresponding to 89.48% of lactose. At the end of maturation, the mixed inoculum showed 92.5% lactose consumption resulting in a cheese with 2.26 g/Kg lactose (Table 1). Right after cheese molding, glucose detected was lower than 0.87 g/Kg in all cheeses and glucose levels at 7 d, 14 d, and 21 d of maturation were also minimal for all cheeses. The glucose detected from 7 d of maturation onward originated from lactose breakdown and glucose consumption likely occurring between sampling intervals, resulting in the low measured concentration of glucose.

Table 1 Concentration (g/Kg) of carbohydrates and acids in cheese over 21 days of maturation.

Inoculum	Time	Lactose	Glucose	Citric Acid	Lactic Acid	Acetic Acid
K. lactis B10	0	27.15 ± 0.05	nd	0.98 ± 0.1	0.20 ± 0.3	1.03 ± 0.0
	7	6.44 ± 0.01	0.19 ± 0.05	0.1 ± 0.00	0.18 ± 0.16	0.17 ± 0.01
	14	4.31 ± 0.01	nd	1.13 ± 0.03	0.10 ± 0.23	0.67 ± 0.06
	21	0.88 ± 0.25	0.73 ± 0.05	1.58 ± 0.1	nd	0.74 ± 0.0
T. delbrueckii B14	0	33.27 ± 0.56	nd	1.26 ± 0.0	0.18 ± 0.01	0.66 ± 0.01
	7	18.1 ± 0.06	nd	0.27 ± 0.02	0.75 ± 0.63	0.16 ± 0.02
	14	8.17 ± 0.26	1.69 ± 0.34	1.01 ± 0.03	4.67 ± 0.24	1.03 ± 0.11
	21	5.21 ± 0.32	0.43 ± 0.06	0.81 ± 0.0	7.61 ± 0.04	0.76 ± 0.07
Mixed inoculum	0	30.06 ± 0.10	0.87 ± 0.12	1.13 ± 0.1	0.16 ± 0.0	0.86 ± 0.0
	7	17 ± 0,25	nd	1.07 ± 0.02	4.33 ± 0.65	0.77 ± 0.03
	14	3.16 ± 0.24	0.19 ± 0.05	0.62 ± 0.03	9.35 ± 0.03	0.67 ± 0.01
	21	2.26 ± 0.0	0.37 ± 0.04	1.46 ± 0.0	6.78 ± 0.0	0.96 ± 0.0

Among the identified acids, lactic acid is mainly responsible for the taste and preservation of cheeses. The acetic acid also contributes to the taste of cheeses providing spicy, vinegar and even fruity and floral odors (Zheng et al., 2017; Matera et al., 2018). After 21 days of maturation, the cheese inoculated with *K. lactis* B10 showed decreased lactic acid and acetic acid concentrations while citric acid content increased from 0.98 g/Kg to 1.58 g/Kg (Table 1). In contrast, cheese produced with *T. delbrueckii*

B14 showed an increasing lactic acid concentration from 0.18 g/Kg to 7.61 g/Kg while decreasing citric acid concentration from 1.26 g/Kg to 0.81 g/Kg (Table 1). Interestingly, in the cheese produced with the mixed inoculum, lactic acid increased to similar levels as cheese produced with *T. delbrueckii* B14, while citric acid content increased as observed in the cheese produced with *K.* lactis B10 (Table 1). According to Matera et al. (2018), the concentrations of lactic, citric, and acetic acids can vary among different types of cheeses depending on different production methods, maturation time, and microorganisms used.

3.8 Analysis of volatile compounds

In general, the concentration of most volatile compounds identified increased over the maturation period (Table 2). At the end of 21 days of maturation, the cheese inoculated with *K. lactis* B10 presented the highest total concentration of acids and alcohols, while cheese produced with mixed inoculum showed the highest ester content (Table 2).

3.8.1 Acids

Total acid content increased over the maturation period (Table 2). After 7 d and 14 d of maturation, cheese produced with mixed inoculum showed the highest total acid concentrations with 87.19 µg/Kg and 526.28 µg/Kg, respectively. After 21 days of maturation, the highest total acid concentration was found in cheese produced with *K. lactis* B10 (Table 2). It is interesting to note that only cheese produced with the mixed inoculum showed all identified acids after 21 days of maturation, with butanoic acid (241.36 µg/Kg), hexanoic acid (233.07 µg/Kg) and decanoic acid (209.25 µg/Kg) were the most abundant acids (Table 2).

The fatty acids present during the cheese maturation process contribute significantly to cheese aroma, either by their aromatic nature or by virtue of being precursors of alcohols, esters, methyl ketones, and other compounds. Hexanoic acid, along with octanoic and decanoic acids, are important contributors to the taste of a wide variety of cheeses (Delgado, González-Crespo, Cava, & Ramírez, 2011). Curioni and Bosset (2002) reported that hexanoic acid is one of the compounds responsible for the characteristic flavor of Grana Padano and Roncal cheeses (goat milk cheeses) produced in Spain, while decanoic and octanoic acids are among the main chemicals responsible for the aroma of Roncal cheese. In general, in our work, cheese produced with *T. delbrueckii* B14 showed a poorer acid profile in terms of diversity and quantity than other cheeses tested (Table 2).

Acids can be obtained from three major routes in the cheese maturation process: lipolysis, where enzymes with lipolytic activities may cause the release of linear chain acids; proteolysis, in which proteolytic enzymes form branched chain acids, and lactose fermentation (Delgado, González-Crespo, Cava, García-Parra, & Ramírez, 2010; Curioni & Bosset, 2002). The results reported above can be correlated with the profiles of sugar consumption (Table 1), in which *K. lactis* B10 and the mixed inoculum were more efficient in lactose consumption compared to *T. delbrueckii* B14. This efficiency resulted in the production of high concentrations of volatile compounds.

3.8.2 Alcohols

A total of 9 alcohols were found in the three cheeses produced (Table 2). The mixed inoculum presented the highest total alcohol content after 7 and 14 days of maturation (Table 2) and also showed the greatest diversity of alcohols (Table 2). The most abundant alcohols found in mixed inoculum cheese after 21 days of maturation

were 1-butanol (237.48 µg/Kg), isoamyl alcohol (990.94 µg/Kg), 2-ethyl-1-hexanol (112.67 µg/Kg), 2,3-butanediol (1336.16 µg/Kg), and 2-phenethylethanol (714.54 µg/Kg) (Table 2). 2,3-Butanediol was the most abundant compound measured in the three produced cheese, except for the mixed inoculum on the 7th day of maturation (Table 2). According to Bertuzzi, McSweeney, Rea, & Kilcawley, (2018), 2,3-butanediol can be produced from residual citrate in cheese curd, which could explain its high concentration in the produced cheeses. 2-Phenethylethanol, isoamyl alcohol, and isobutanol were other alcohols also found in high concentrations and are important to cheese aroma. 2-Phenethylethanol is associated with pleasant notes of roses (Hayaloglu et al., 2013; Zheng et al., 2017) while isoamyl alcohol is considered one of the main cheese flavor producers, providing a sweet and slightly fresh taste (Nogueira, Lubachevsky, & Rankin, 2005). Isoamyl alcohol and isobutanol are both associated with fruity, alcoholic, pomace, and fusel flavors (Sørensen, Gori, Petersen, Jespersen, & Arneborg, 2011).

Although individual compounds showed varying changes in concentration after 21 days of maturation (Table 2), the overall total alcohol content increased with maturation for all produced cheeses. The increase in compound concentrations during maturation is a result of the biochemical reactions that occur, with increasing reaction levels resulting in a greater number of volatile compounds and higher amounts (Delgado et al., 2010). However, some alcohols may show decreasing levels during maturation, may be related to the reaction of alcohols with acids during ester formation (Delgado et al., 2011).

3.8.3 Esters

Esters were the group of compounds with the greatest diversity in the produced cheeses (Table 2). These compounds can be formed by the esterification of acids and alcohols or by the metabolism of amino acids (Hayaloglu et al., 2013; Bezerra et al., 2017). Among the esters, ethyl esters have the greatest impact on cheese aroma (Pinho et al., 2003; Bezerra et al., 2017) and generally increased in concentration during maturation. Phenethyl acetate was the most abundant ester in all cheeses at all maturation timepoints except for at 21 d of maturation of cheese produced with *T. delbrueckii* B14. The highest concentration of phenethyl acetate (2708.82 µg/Kg) was measured in the cheese produced by mixed inoculum at 21 days of maturation (Table 2). Phenethyl acetate is one of the main esters in cheese, providing a pleasant, sweet, herbaceous odor and is commonly found in Camembert cheeses (Curioni & Bosset, 2002; Sadecka, Kolek, Pangallo, Valík, & Kuchta, 2014). Ethyl octanoate was also found in high concentrations after 21 d of maturation for all produced cheeses, with 286.70 µg/Kg of ethyl octanoate measured in the cheese produced by *T. delbrueckii* B14 (Table 2). Ethyl octanoate imparts a pleasant, fresh, clove-like, and sweet odor to cheeses.

Besides providing sweet and floral notes to the final product (Zheng et al., 2017), esters are also desirable volatile compounds because they have high volatility and fruity aromas, which may help to mask undesirable aromas of acids, aldehydes and amines.

3.8.4 Aldehydes

Three aldehydes, heptanal, octanal and nonanal, were found in cheeses produced (Table 2), with only nonanal found in all cheeses. Linear chain aldehydes like nonanal are common and important precursors of aromas in cheese. In general, aldehydes are found in low concentrations in cheese because they are rapidly reduced to primary

alcohols or acids. For this reason, aldehyde retention in cheese is usually temporary and the low aldehyde concentrations can be indicators of an ideal maturation (Curioni & Bosset, 2002; Delgado et al., 2010). Although aldehydes are not found in high concentrations in cheese, they likely play an important role in the flavor of the final product (Zheng et al., 2017).

3.8.5 Other volatile compounds

Other volatile compounds, including ketones, were found in the produced cheese (Table 2). Among them, acetoin was the most abundant in all cheeses produced, which provides a "buttery and creamy" flavor and a "fermented milk" aroma to the cheese (Curioni & Bosset, 2002; Matera et al., 2018). The acetoin content is also related to the 2,3-butanediol levels, as this alcohol is produced by many yeasts utilizing acetoin as an intermediate.

Phenol can contribute significantly to the aroma of the cheese with descriptors such as sweet, caramel, smoky and charred (Curioni & Bosset, 2002). It can be formed during the decomposition of tyrosine by yeasts and bacteria (Curioni & Bosset, 2002; Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994).

Table 2
Concentration (µg/Kg) of the volatile compounds as determined by HS SPME GC-MS in cheese over 21 days of maturation

Nº	Compounds	Concentration									Descriptors
		K. lactis B10			*T. delbrueckii* B14			Mixed inoculum			
		T7	T14	T21	T7	T14	T21	T7	T14	T21	
	Acids										
1	Hexanoic acid	1.8 ± 1.27	5.00 ± 3.54	233.9 ± 0.0	nd	nd	174.11 ± 0.88	15.95 ± 5.27	nd	233.07 ± 2.17	Stale, butter, sour, fruity, pungent (Kilcawley, 2017)
2	Isocaproic acid	nd	nd	675.04 ±7.32	nd	nd	nd	nd	232.00 ± 8.09	57.65 ± 1.53	
3	Decanoic acid	14.28 ± 8.67	60.4 ± 0,50	114.2 ± 0.74	nd	262.91 ± 5.85	197.48 ± 9.63	38.16 ± 2.61	160.94 ± 4.06	209.25 ± 2.57	Stale, butter, sour, fruity, pungent (Kilcawley, 2017)
4	Isobutanoic acid	nd	nd	nd	nd	nd	nd	2.71 ± 0.89	nd	53.39 ± 7.00	Cheese, rancid, butter (Kubícková and Grosch 1997)
5	Butanoic acid	nd	nd	nd	nd	nd	nd	nd	59.94 ± 4.77	241.36 ± 0.82	Sweaty, butter, cheese, strong, acid, fermented odour (Kilcawley, 2017 Sádecká et al., 2014)
6	Undecanoic acid	13.89 ± 9.82	13.85 ± 9.79	nd	nd	nd	nd	30.37 ± 0.03	73.40 ± 0.58	89.84 ± 6.90	
	Total acids	**29.97**	**79.25**	**1023.14**	**0**	**262.91**	**371.59**	**87.19**	**526.28**	**884.56**	
	Alcohol										
7	Isobutanol	31.18 ± 0.00	22.62 ± 3.09	113.47 ± 4.75	6.15 ± 1.67	19.64 ± 6.95	94.97 ± 1.65	28.66 ± 9.47	68.22 ± 9.05	49.10 ± 8.66	
8	1-Butanol	nd	2.19 ± 1.55	nd	nd	18.54 ±5.48	79.89 ± 3.11	nd	5.37 ± 7.59	237.48 ± 5.85	Floral, fragrant, fruity, sweet (Singh, Drake and Cadwallader, 2003)
9	Isoamyl alcohol	nd	nd	nd	142.22 ± 4.05	212.95 ± 6.49	232.76 ± 6.71	332.96 ± 1.89	811.02 ± 4.89	990.94 ± 5.85	Fresh cheese, breath-taking, alcoholic, fruity, grainy (Kilcawley, 2017)
10	Hexanol	nd	9.93 ± 7.02	nd	nd	53.35 ± 1.28	30.41 ± 1.50	11.98 ± 3.95	35.74 ± 0.71	19.70 ± 7.85	Fatty, floral, green (Singh et al., 2003)
11	2-Ethy-l-hexanol	nd	40.73 ± 7.98	nd	nd	57.03 ± 5.02	57.03 ± 3.43	450.68 ± 8.45	50.30 ± 1.13	112.67 ± 9.34	Animal, cardboard (Thomsen et al., 2012)
12	2,3-Butanediol	246.11 ± 0.43	1047.39 ± 8.12	3293.66 ± 1.08	162.04 ± 2.03	656.46 ± 5.01	1262.43 ± 5.92	99.17 ± 7.68	692.17 ± 7.74	1336.16 ± 4.73	Fruity (Singh et al., 2003)
13	1,3-Butanediol	nd	4.28 ± 0.00	18.45 ± 4.17	nd	9.44 ± 6.67	27.63 ± 2.73	2.74 ± 5.09	3.66 ± 5.17	45.83 ± 9.97	
14	Phenethyl alcohol	13.08 ± 4.08	103.08 ± 6.21	280.49 ± 0.39	nd	58.34 ± 1.29	85.08 ± 9.14	24.66 ± 8.14	37.63 ± 1.03	714.54 ± 8.48	Unclean, rose, violet-like, honey, floral (Kilcawley, 2017)
15	1-Nonanol	nd	5.55 ± 0.75	nd	nd	11.54 ± 4.38	nd	30.21 ± 9.98	nd	nd	
	Total alcohols	**290.37**	**1235.77**	**3706.07**	**310.41**	**1097.29**	**1986.29**	**981.06**	**1704.11**	**3506.42**	

	Esters										
16	Ethyl butanoate	nd	nd	25.25 ± 9.02	nd	nd	22.80 ± 6.11	nd	134.50 ± 5.31	35.30 ± 1.28	Bubble gum, fruity (Singh et al., 2003)
17	Isoamyl acetate	nd	4.38 ± 3.09	76.39 ± 3.45	nd	23.54 ± 6.64	104.04 ± 6.32	7.06 ± 2.33	58.76 ± 5.31	534.15 ± 1.28	Fruity, banana, candy, sweet (Barron et al., 2005; Curioni & Bosset,2002; Qian, Burbank and Wang, 2006)
18	Ethyl hexanoate	nd	1.41 ± 1.00	20.65 ± 1.21	nd	45.08 ± 5.76	132.97 ± 2.65	nd	5.14 ± 1.71	31.10 ± 9.45	Fruity, pineapple, sweet, banana (Kilcawley, 2017; Singh et al., 2003)
19	Amyl hexanoato	8.58 ± 6.07	nd	7.19 ± 5.08	nd	13.72 ± 9.70	20.46 ± 4.46	nd	64.22 ± 4.97	129.00 ± 6.75	
20	Ethyl octanoate	nd	13.71 ± 6.16	37.60 ± 0.31	nd	50.80 ± 1.28	286.70 ± 3.87	16.96 ± 5.60	37.36 ± 1.10	119.37 ± 1.11	Pear, apricot, sweet, fruity, banana, pineapple (Kilcawley, 2017; Singh et al., 2003)
21	Ethyl decanoate	nd	4.99 ± 0.47	13.42 ± 6.32	nd	45.70 ± 5.65	14.58 ± 9.00	5.05 ± 1.66	5.31 ± 7.50	28.81 ± 0.74	Pineapple, sweet, fruity, banana (Kilcawley, 2017)
22	Amyl butanoate	nd	11.02 ± 7.79	8.08 ± 5.71	nd	nd	nd	nd	nd	10.89 ± 3.64	
23	Isobutyl propionate	nd	nd	nd	nd	nd	nd	nd	nd	9.65 ± 2.78	
24	Phenethyl acetate	35.57 ± 3.26	214.05 ± 6.58	661.94 ± 1.89	19.07 ± 7.92	190.11 ± 2.43	269.55 ± 9.96	706.58 ± 3.52	619.35 ± 3.00	2708.82 ± 8.55	Floral, rose-like (Kubícková and Grosch 1997, Kubíncková and Grosch 1998)
25	Phenethyl propionate	nd	nd	nd	nd	nd	nd	17.11 ± 5.65	8.72 ± 2.33	258.54 ± 5.63	
26	Phenethyl butyrate	nd	nd	nd	nd	nd	nd	nd	12.44 ± 7.66	35.57 ± 5.87	Floral, fruity (Kilcawley, 2017)
27	Isoamyl propianate	nd	nd	nd	nd	nd	nd	nd	nd	76.93 ± 8.79	
28	Butyl acetate	nd	nd	nd	nd	nd	nd	nd	31.01 ± 0.80	1.89 ± 2.67	Pear, ethereal, green (Barron et al., 2005)
29	Butyl butanoate	nd	nd	nd	nd	20.36 ± 4.39	nd	nd	33.65 ± 9.85	37.33 ± 2.79	Pineapple, banana, sweet (Barron et al., 2005)
	Total esters	**44.15**	**249.56**	**850.52**	**19.07**	**358.19**	**882.22**	**752.76**	**1010.46**	**4017.35**	
	Aldehydes										
30	Nonanal	2.46 ± 6.89	6.38 ± 5.36	nd	16.44 ± 9.58	nd	20.05 ± 6.84	14.52 ± 4.79	13.95 ± 6.11	28.82 ± 8.58	Green (Singh et al., 2003)
31	Heptanal	nd	nd	nd	nd	nd	nd	5.02 ± 1.65	4.51 ± 6.37	nd	Fatty, oily, green (Singh et al., 2003)
32	Octanal	nd	nd	nd	nd	nd	nd	8.34 ± 2.75	16.85 ± 5.08	7.00 ± 9.90	Green, fatty, soapy, fruity, orange peel (Singh et al., 2003)
	Total aldehydes	**2.46**	**6.38**	**nd**	**16.44**	**nd**	**20.05**	**27.88**	**35.31**	**35.82**	

#											
	Others										
33	Acetoin	167.78 ± 5.26	193.52 ± 2.14	817.56 ± 7.90	143.60 ± 7.23	354.75 ± 4.93	566.92 ± 1.86	183.00 ± 0.48	512.92 ± 0.49	339.84 ± 7.10	Sour milk (Arora, Cormier, and Lee 1995)
34	Phenol	38.25 ± 3.93	66.99 ± 4.14	248.24 ± 2.32	nd	198.34 ± 7.00	155.13 ± 7.36	157.74 ± 2.13	100.72 ± 7.18	251.78 ± 8.99	Medicinal (Singh et al., 2003)
35	Butylacetone	nd	nd	nd	nd	13.81 ± 9.76	34.38 ± 4.31	nd	nd		
36	2-Acetoxy-3-butanone	nd	2.46 ± 1.74	19.46 ± 4.23	nd	7.63 ± 2.24	30.19 ± 5.09	2.45 ± 0.80	16.85 ± 8.90	58.97 ± 3.40	
37	2-Nonanone	nd	5.87 ± 2.17	15.95 ± 0.03	4.95 ± 3.49	39.18 ± 7.17	27.03 ± 9.50	7.89 ± 2.60	14.14 ± 5.10	27.95 ± 0.44	Green, earthy, blue cheese, fatty, fruity, musty, varnish, malty, hot milk (Kilcawley, 2017; Singh et al., 2003)
38	Dodecane	nd	9.93 ± 1.48	nd	nd	nd	20.85 ± 2.31	3.68 ± 1.21	6.76 ± 9.56	10.02 ± 5.96	
	Total others	**206.03**	**278.77**	**1101.21**	**148.55**	**613.71**	**834.5**	**354.76**	**651.39**	**688.56**	

nd- Not detected.

4 Conclusions

T. delbrueckii B14 and *K. lactis* B10 showed high survival rates of simulated gastrointestinal conditions and some adaptation to different NaCl concentrations and temperatures. When used as single or mixed inoculum, these yeasts remained viable during 21 days of cheese maturation, generating cheeses with different profiles of aromatic volatile compounds. For the mixed inoculum cheeses, a greater abundance of acids, alcohols and esters was observed, indicating a synergistic effect between *K. lactis* B10 and *T. delbrueckii* B14 during cheese maturation. The obtained results thus demonstrate that *T. delbrueckii* B14 and *K. lactis* B10 are interesting alternatives to utilize to produce potentially functional cheeses with varying aromatic characteristics. However, to confirm the benefits of these yeasts as probiotics, further experiments should be performed, especially under in vivo conditions.

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ANEXO – PATENTE

PATENTE - Utilização de leveduras potencialmente probióticas como culturas iniciadoras para elaboração de queijo com baixo teor ou sem lactose

Patente apresentada como permitido pelo NPI – Instituto Nacional de Propriedade Industrial

RELATÓRIO DESCRITIVO

Utilização de leveduras potencialmente probióticas como culturas iniciadoras para elaboração de queijo com baixo teor ou sem lactose

Campo técnico da invenção

[01] A presente invenção refere-se à utilização de leveduras potencialmente probióticas como culturas iniciadoras para produção de queijo com baixo teor ou sem lactose.

Fundamentos da invenção

[02] A busca por produtos com baixo teor ou sem lactose tem crescido nos últimos anos em função, principalmente, da intolerância à lactose apresentada pelos consumidores em todo mundo. De maneira similar, constata-se também a demanda por produtos considerados funcionais, ou seja, aqueles que contêm microrganismos denominados probióticos. Para atendimento destas demandas, a elaboração de produtos com baixo teor ou sem lactose, como no caso dos queijos, é realizada com o uso da enzima b-galactosidase, obtida após extração a partir das células microbianas. No caso dos produtos contendo probióticos, os microrganismos frequentemente empregados são em sua grande maioria bactérias, principalmente aquelas do gênero Lactobacillus. Leveduras como Kluyveromyces lactis são reconhecidas como produtoras da enzima b-galactosidase e são em alguns casos utilizadas como culturas iniciadoras. No entanto, não há registro/relato ou proteções intelectuais que descrevam o uso de leveduras, especificamente das espécies *Torulaspora delbrueckii* e *Kluyveromyces lactis* isoladas de queijo, e, que além de potencialmente probióticas, sejam eficientes na quebra da lactose para uso como culturas iniciadoras na produção de queijos com características funcionais e com baixo teor ou sem lactose. A utilização de uma cultura iniciadora potencialmente probiótica e eficiente produtora de b-galactosidade com consequente consumo de lactose constitui uma tecnologia ainda não explorada, caracterizando o principal aspecto inovador da presente patente. Esta tecnologia, além de apresentar potencial para atendimento de um nicho de mercado crescente, pode impactar na redução de custos do processo de produção de queijo com baixo teor ou sem lactose quanto ao consumo da enzima b-galactosidase.

[03] Na patente US6902749 (B1) é proposto o uso da levedura *Kluyveromyces* lactis assim como em nossa proposta. No entanto, nesta patente US6902749 (B1), os autores utilizam a levedura na forma "atenuada" para a fabricação de queijo, análogos de queijo e produtos

derivados de queijo. Segundo a definição apresentada na patente, "atenuação" significa que a população da levedura antes de ser utilizada na produção de queijo foi submetida a um tratamento que resultou na morte de preferencialmente 99% das células. Ainda segundo a proposta dos autores, a atenuação das células deve ser preferencialmente feita utilizando-se micro-ondas. Esta utilização de uma cultura atenuada de leveduras faz com que esta patente seja diferente de nossa proposta, uma vez que apresentamos o uso de uma cultura iniciadora de *Kluyveromyces lactis* com, preferencialmente, a totalidade de células da população viável e não morta, como na patente US6902749 (B1). A presente proposta ainda difere da patente US6902749 (B1) quanto ao foco no efeito de redução da lactose resultante do metabolismo da levedura ativa utilizada, quanto ao uso de uma levedura *Kluyveromyces lactis* e também de uma levedura *Torulaspora delbrueckii,* ambas caracterizadas como potencialmente probióticas.

[04] Na US2016165912 (A1), reporta-se como principal foco a produção de queijo com flavor característico de queijos de cabra ou ovelha. Ainda nesta patente, tem-se como reivindicações o uso de uma levedura lipolítica específica, *Yarrowia lipolytica* depositada na coleção de cultura BCCM/IHEM sob número IHEM 26011 e também o uso de uma levedura *Kluyveromyces lactis* especifica depositada na coleção de cultura BCCM/IHEM sob número IHEM 26012. Estes aspectos descritos da patente US2016165912 (A1) a tornam diferente desta proposta uma vez que visamos o uso de uma cultura iniciadora de *Kluyveromyces lactis* que não a IHEM 26012 e, sendo ainda a levedura desta proposta caracterizada como potencialmente probiótica. A presente patente difere também da patente US2016165912 (A1) pois visa a obtenção de queijo com baixo teor de lactose devido ao uso das leveduras como culturas iniciadoras. Ainda, diferente da patente US2016165912 (A1), que propõe o uso da levedura *Yarrowia lipolytica,* propõe-se, além da *Kluyveromyces lactis*, o uso da levedura *Torulaspora delbrueckii.*

[05] Na patente WO 2015120123 A1 aborda o uso de fungos do Reino Fungi para acidificação do leite e métodos para produção de produtos lácteos com leite acidificado com pelos menos um microrganismo do Reino Fungi. Embora nesta patente seja reivindicado o uso de leveduras como *Kluyveromyces lactis* na acidificação do leite para fabricação de produtos lácteos incluindo queijos, esta proposta difere amplamente de nossa patente uma vez que o foco de nossa tecnologia não diz respeito à acidificação do leite e, sim à redução do teor de lactose do produto final (queijo) a partir do metabolismo deste açúcar por leveduras produtoras de b-galactosidase, especificamente *Kluyveromyces lactis* e *Torulaspora delbrueckii*. Ressalta-se também que nossa tecnologia diferencia da patente

WO 2015120123 devido ao fato de propormos o uso de leveduras que são especificamente caracterizadas como potencialmente probióticas. Essa característica é inovadora no contexto do pedido de proteção e, juntamente com a redução do teor de lactose constituem dois pontos principais de nossa proposta e que não são contemplados pela patente WO 2015120123. Em suma, enquanto a patente WO 2015120123 A1 reporta o uso de microrganismos do Reino Fungi em processo destinado claramente à acidificação do leite e suas consequências na produção de queijo, nossa tecnologia e processo tem como principal objetivo a produção de queijo que reúne as características baixo teor ou sem lactose empregando-se leveduras potencialmente probióticas de modo a se obter um produto potencialmente funcional.

Sumário da invenção

[06] A presente invenção apresenta-se como uma inovação no uso de culturas iniciadoras potencialmente probióticas para produção de queijos com baixo teor ou sem lactose. Considerando a crescente demanda mundial por produtos funcionais e também por produtos com baixo teor ou sem lactose, a tecnologia de "utilização de leveduras potencialmente probióticas como culturas iniciadoras para elaboração de queijo com baixo teor ou sem lactose", traz benefícios significativos ao campo da produção de queijos com culturas iniciadoras por: possibilitar a produção de queijos de modo atender a demanda de um nicho de mercado consumidor voltado para produtos com baixo teor ou sem lactose sem a utilização direta da enzima b-galactosidase; e possibilitar a produção queijos potencialmente funcionais obtidos sem o uso de bactérias probióticas e com características peculiares de textura, aroma e sabor proporcionados exclusivamente por leveduras.

[07] A invenção "utilização de leveduras potencialmente probióticas como culturas iniciadoras para elaboração de queijo com baixo teor ou sem lactose" inclui o uso de leveduras, especificamente *Kluyveromyces lactis* e *Torulaspora delbrueckii* isoladas de queijo, caracterizadas como potencialmente probióticas e eficientes na quebra da lactose durante a fabricação de queijos quando utilizadas como inoculo simples de *Kluyveromyces lactis* e *Torulaspora delbrueckii*; ou como inoculo misto de *Torulaspora delbrueckii* no leite para elaboração de queijo. O leite utilizado para fabricação do queijo pode ser pasteurizado ou cru. A inoculação deve ser realizada utilizando-se as leveduras na sua forma seca ativa ou fresca de modo a resultar em populações de pelos menos 10^4 células ou unidades formadoras de colônias para cada mililitro de leite. Como cultura iniciadora pode-se empregar a *Kluyveromyces lactis* pura, *Torulaspora delbrueckii* pura, ambas em

cultura mista; ou estas 3 formas ainda combinadas com culturas de bactérias empregadas na produção de queijo. O termo "bactérias" aqui utilizado refere-se a qualquer cultura iniciadora ou não iniciadora que possa ser empregada na fabricação de queijo. As leveduras devem ser inoculadas concomitante ou logo anteriormente à adição do coagulante quando este for utilizado. As leveduras podem ser utilizadas sob as condições de pH, temperatura e concentração de sal comumente empregadas na produção de queijos.