GABRIELLY CARVALHO ANDRADE

POTENCIAL PROBIÓTICO DE *KLUYVEROMYCES LACTIS* E *TORULASPORA DELBRUECKII* COMO CULTURAS INICIADORAS NA PRODUÇÃO DO QUEIJO

LAVRAS – MG

2021

GABRIELLY CARVALHO ANDRADE

POTENCIAL PROBIÓTICO DE *KLUYVEROMYCES LACTIS* E *TORULASPORA DELBRUECKII* COMO CULTURAS INICIADORAS NA PRODUÇÃO DO QUEIJO

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.

Prof. Dr. Whasley Ferreira Duarte
Orientador

LAVRAS – MG

2021

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Andrade, Gabrielly Carvalho.
Potencial probiótico de *Kluyveromyces lactis* e *Torulaspora delbrueckii* como culturas iniciadoras na produção de queijo / Gabrielly Carvalho Andrade. - 2021.
79 p. : il.

Orientador(a): Whasley Ferreira Duarte.

Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2021.
Bibliografia.

1. Leveduras. 2. Inóculo misto. 3. Maturação de queijos. I. Duarte, Whasley Ferreira. II. Título.

GABRIELLY CARVALHO ANDRADE

POTENCIAL PROBIÓTICO DE *KLUYVEROMYCES LACTIS* E *TORULASPORA DELBRUECKII* COMO CULTURAS INICIADORAS NA PRODUÇÃO DO QUEIJO

PROBIOTIC POTENTIAL OF *KLUYVEROMYCES LACTIS* AND *TORULASPORA DELBRUECKII* AS STARTER CULTURES IN CHEESE PRODUCTION

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.

APROVADA em 27 de maio de 2021

Prof. Dr. Flaviano dos Santos Martins UFMG

Prof.ª. Dra. Patrícia Gomes Cardoso UFLA

Prof. Dr. Whasley Ferreira Duarte
Orientador

LAVRAS – MG

2021

Dedico este trabalho a Deus, por mais esta vitória alcançada e à minha família pelo apoio e carinho em todos os momentos desta trajetória.

AGRADECIMENTOS

Primeiramente agradeço a Deus, por essa dádiva alcançada, por sentir sempre de perto sua mão forte me guiando e me sustentando ao longo desta jornada.

Aos meus pais, Emiliane e Aloísio, pelo amor incondicional que sempre me apoiou nos estudos e nas escolhas tomadas, à minha irmã Adrielly, seu esposo Junio, pelas palavras de incentivo nos momentos difíceis e pelas orações diárias para que mais essa etapa fosse concluída.

Agradeço ao prof. Dr. Whasley Ferreira Duarte, por todo aprendizado, paciência e contribuição para a minha formação acadêmica.

À Rafaela Pereira Andrade, pela ajuda, amizade e suporte durante todo o percurso para a execução deste trabalho.

À Equipe do Laboratório de Microbiologia, pelo apoio e carinho cotidiano nesta trajetória.

Aos meus amigos, pela torcida para essa conquista!

Ao programa de pós-graduação em Microbiologia Agrícola, a secretária Rose pela receptividade, atenção e carinho.

Aos professores por todo o conhecimento transmitido, em especial a prof.ª Dra. Roberta Hilsdorf Piccoli por toda a atenção, disponibilidade e colaboração.

À Banca Examinadora por fazer parte deste momento.

À Universidade Federal de Lavras.

O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

MUITO OBRIGADA!

RESUMO GERAL

O queijo é um dos produtos lácteos mais consumidos do mundo, sendo um alimento altamente nutritivo, saboroso e constituído por uma rica diversidade microbiana que o caracteriza em diversos tipos. Neste contexto, tem se destacado as leveduras, devido a sua interação e prevalência em produtos lácteos, que contribuem nos processos de maturação e fermentação. Além disso, as leveduras apresentam características interessantes que as tornam potenciais candidatas probióticas e o queijo pode ser um bom carreador para estes microrganismos. No presente trabalho, objetivou-se produzir queijos utilizando as duas leveduras *Kluyveromyces lactis* e *Torulaspora delbrueckii*, previamente isoladas do queijo Canastra por meio de dois tratamentos, inserindo-as na superfície e massa do queijo, avaliando sua influência nas características físico-químicas e sensoriais que contribuem para os aspectos organolépticos do produto final. Os queijos produzidos foram avaliados quanto aos seus aspectos intrínsecos e extrínsecos por meio de análises microbiológicas, texturais, sensoriais e produção de enzimas extracelulares. Testes *in vivo* e *in vitro* foram realizados para validar se as leveduras em estudo são seguras para serem ingeridas. Os isolados foram submetidos ao método de preservação por liofilização, avaliados quanto as condições de armazenamento em temperatura ambiente e de 4°C durante 120 dias. Posteriormente, simulados a passagem pelo trato gastrointestinal, afim de serem utilizadas como suplemento probiótico ou cultura starter na indústria alimentícia. As leveduras permaneceram viáveis ao longo do processo de maturação dos queijos, com populações respectivamente de 10^8 UFC/g e 10^7 UFC/g. Quando inoculados na massa, os parâmetros texturais apresentaram menor dureza e mastigabilidade, ressaltando que esses fatores interferem na aceitação do produto pelo consumidor, tendo assim melhor aceitabilidade sensorial. Os resultados obtidos mostraram que *K. lactis* B10 e *T. delbrueckii* B14, são leveduras interessantes no contexto de probióticos devido à ausência de atividade hemolítica, gelatinase, DNAse e degradação da mucina, sendo considerados um pré-requisito de segurança para a seleção de uma linhagem probiótica. Nas condições *in vivo* apresentaram alta sobrevivência no trato gastrointestinal. Além disso, a levedura *K. lactis* B10, quando administrada em camundongos, resultou em maior sobrevida, após desafio com *Salmonella enterica* subsp *enterica* sorovar Typhimurium. As leveduras não apresentaram atividade proteolítica, apenas atividade lipolítica que atua na melhoria do sabor de produtos lácteos, resultando em uma maior apreciação sensorial. Os isolados liofilizados mantiveram estáveis durante todo o processo ao serem condicionados apenas na temperatura de 4°C. As contagens de placas mostraram que a viabilidade da temperatura de 4°C foi de 10^5 UFC/ml ao final do processo, enquanto que na temperatura ambiente aos 60 dias a população foi de 10^4 UFC/ml. As leveduras liofilizadas quando submetidas as condições quimicamente simuladas do trato gastrointestinal mantiveram viáveis. Esses resultados demonstram que o método de preservação utilizado foi adequado mantendo as propriedades químicas, físicas e microbiológicas fornecendo ao mercado consumidor um suplemento probiótico.

PALAVRAS-CHAVE: Leveduras. Inóculo misto. Maturação de queijos.

GENERAL ABSTRACT

Cheese is one of the most consumed dairy products in the world, being a highly nutritious, tasty food and constituted by a rich microbial diversity that characterizes it in several types. In this context, yeasts have stood out due to their interaction and prevalence in dairy products that contribute to the maturation and fermentation processes. The yeasts have interesting characteristics that make them potential probiotic candidates and cheese can be a good carrier for these microorganisms. The present work aimed to produce cheeses using the two yeasts *Kluyveromyces lactis* and *Torulaspora delbrueckii*, previously isolated from Canastra cheese using two treatments, inserting them into the surface and mass of the cheese, evaluating their influence on the physical-chemical and sensory characteristics that contribute to the organoleptic aspects of the final product. The cheeses produced were evaluated for their intrinsic and extrinsic aspects through microbiological, textural, sensory analysis, production of extracellular enzymes. *In vivo* and *in vitro* tests were carried out to validate whether the potential yeasts under study are safe to be ingested. The isolates were subjected to the preservation method by freeze-drying, evaluated for storage conditions at room temperature, and in the 4°C for 120 days. Subsequently simulated the passage through the gastrointestinal tract, to be used as a probiotic supplement or starter culture in the food industry. Yeasts remained viable throughout the cheese maturation process, with populations of 10^8 CFU / g and 10^7 CFU / g, respectively. When inoculated into the dough, the textural parameters showed less hardness and chewability, emphasizing that these factors interfere with the acceptance of the product by the consumer, thus having better sensory acceptability. The results obtained showed that *K. lactis* B10 and *T. delbrueckii* B14 are interesting yeasts in the context of probiotics due to the absence of hemolytic activity, gelatinase, DNAse, and mucin degradation being considered a safety prerequisite for the selection of a probiotic strain. Under *in vivo* conditions, they showed high survival in the gastrointestinal tract. Furthermore, the yeast *K. lactis* B10, when administered to mice, resulted in greater survival after challenge with *Salmonella enterica* subsp *enterica* serovar Typhimurium. Yeasts did not show proteolytic activity and only lipolytic activity that improves the taste of dairy products resulting in a greater sensory appreciation. The lyophilized isolates remained stable throughout the process by being conditioned only at a temperature of 4 ° C. The plate counts showed that the viability of the 4 ° C temperature was 10^5 UFC / ml at the end of the process, while at room temperature at 60 days the population was 10^4 UFC / ml. Lyophilized yeasts when submitted to chemically simulated conditions of the gastrointestinal tract remained viable. These results demonstrate that the preservation method used was adequate, maintaining the chemical, physical and microbiological properties, providing the consumer market with a probiotic supplement.

KEYWORDS: Yeasts. Mixed inoculum. Cheese ripening.

LISTA DE ILUSTRAÇÕES

ARTIGO 1

ARTIGO 2

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

SUMÁRIO

PRIMEIRA PARTE- INTRODUÇÃO GERAL

1. INTRODUÇÃO

O queijo é um produto lácteo produzido desde os tempos mais remotos constituindo uma diversidade de formas e sabores, conquistando espaço na culinária tradicional em âmbito mundial. A conversão do leite em queijo é arcaica, constituída pela concentração de lipídeos e proteínas, formando a coalhada (PAULA, CARVALHO, FURTADO; 2009).

A denominação queijo é dada, segundo o Regulamento Técnico de Identidade e qualidade de queijo, descrito pela portaria n° 146 em 1996, como o "produto fresco ou maturado que se obtém por separação parcial do soro do leite ou leite reconstituído (integral, parcial ou totalmente desnatado), ou de soros lácteos, coagulados pela ação física do coalho, de enzimas e bactérias especificas, ácido orgânicos, isolados ou combinados, todos de qualidade apta para uso alimentar, com ou sem agregação de substâncias alimentícias e/ou especiarias e/ou condimentos, aditivos especificamente indicados, substâncias aromatizantes e matérias corantes" (BRASIL, 1996).

As variedades de queijos são obtidas devido as diferentes formas de processamento, que interferem diretamente nas propriedades químicas e físicas, somadas aos aspectos microbiológicos. Estes aspectos, resultam em características sensoriais únicas, que são influenciadas pelas condições intrínsecas, ou seja, como essas comunidades microbianas se estruturam e relacionam, ligadas aos fatores ambientais que permitem o desenvolvimento de populações distintas de microrganismos atuando como o componente-chave da produção (WOLFE *et al*., 2014). O conhecimento da diversidade microbiana do queijo é de suma importância para a qualidade da produção, visando atender e satisfazer as necessidade do mercado consumidor (GIELLO *et al*., 2017).

A microbiota do queijo é representada principalmente pelo grupo das bactérias ácido-láticas, como também fungos leveduriformes, sendo amplamente estudados, quer seja por propriedades específicas, como a inibição que estes propiciam a outros microrganismos patogênicos, ou ao seu potencial probiótico (WOLFE *et al*., 2014).

Um microrganismo é denominado probiótico, quando ao ingeri-lo vivo, regularmente em quantidades adequadas, exercem uma influência positiva para a saúde do hospedeiro (HILL *et al*., 2014). Caracterizam-se por atuarem no intestino humano participando da dinâmica da microbiota local e exercendo atividades benéficas como estabilização da microbiota intestinal, resistência a patógenos e regulador do sistema imunológico fora do intestino (MATHARA *et al*., 2008; CZERUCKA *et al.*, 2007).

Para ser classificado como probiótico, o microrganismo deve possuir algumas características que são cruciais, tais como resistir às condições adversas do trato gastrointestinal (TGI), resultando em efeitos benéficos ao indivíduo como, fortalecer as atividades imunológicas, como melhorias na saúde intestinal, intolerância à lactose, sintomas digestivos desconfortáveis, redução de riscos de diarreia associados a uso de antibióticos e enterocolite necrosante (TYAGI, PRASAD, 2015; HILL *et al*., 2014; ROWLAND *et al*., 2010; FULLER, 1989).

Portanto, o objetivo do nosso estudo foi avaliar a utilização de duas leveduras, *Kluyveromyces lactis* e *Torulaspora delbrueckii*, previamente isolada do queijo Canastra, como culturas iniciadoras na produção de queijos, a fim de compreender suas atribuições aos aspectos organolépticos do produto avaliando sua influência nas características físico-químicas e sensoriais do queijo. Também foi avaliado o potencial probiótico por meio de análises *in vivo* e *in vitro* para validar se as leveduras em estudo, são seguras para serem ingeridas. Os isolados foram submetidos ao método de preservação por liofilização, avaliados quanto as condições de armazenamento em temperatura ambiente e a 4°C durante 120 dias. Posteriormente simulados a passagem pelo TGI, afim de serem utilizados como suplemento probiótico ou cultura starter na indústria alimentícia.

2. REFERENCIAL TEÓRICO

2.1 Produção mineira de queijo

O queijo é definido, segundo o Regulamento Técnico de Identidade e qualidade de queijo, descrito pela portaria n° 146 em 1996, como um "*produto fresco ou maturado que se obtém por separação parcial do soro do leite ou leite reconstituído (integral, parcial ou totalmente desnatado), ou de soros lácteos, coagulados pela ação física do coalho, de enzimas e bactérias especificas, ácido orgânicos, isolados ou combinados, todos de qualidade apta para uso alimentar, com ou sem agregação de substâncias alimentícias e/ou especiarias e/ou condimentos, aditivos especificamente indicados, substâncias aromatizantes e matérias corantes*" (BRASIL, 1996).

No ranking de produtividade mundial de leite, o Brasil se destaca como o quarto maior produtor, produzindo 35,1 bilhões de litros/ ano. Do total produzido, 24,3 bilhões são destinados para processamento pelas indústrias de laticínios que funcionam com serviços de inspeção sanitária e o restante, são destinados ao consumo próprio e à produção artesanal de queijos e derivados (EMBRAPA GADO DE LEITE, 2018).

O queijo se destaca como um dos produtos lácteos mais apreciados no país, sendo que de 3 litros de leite produzidos, 1 L é destinado à fabricação do mesmo. Em 2017, a produção queijeira atingiu 1 milhão de toneladas, gerando a movimentação de aproximadamente R$ 18 bilhões por ano (EMBRAPA GADO DE LEITE, 2018).O governo aplicou no setor mineiro R$ 3 milhões, destinados a aprimorar a produção de queijo Minas Artesanal a fim de fomentar novas pesquisas sobre o processamento do produto.

A produção de queijos artesanais em Minas Gerais distribui-se em 503 municípios, detendo aproximadamente 30 mil produtores. A produção diária de queijo artesanal situa-se em torno de 85,5 mil kg, sendo que nas regiões típicas, há em média 9.500 produtores, destacando-se sete regiões, Araxá, Campo das Vertentes, Canastra, Cerrado, Serra do Salitre, Serro e Triângulo Mineiro, as quais são responsáveis por 35 mil toneladas ao ano, gerando um faturamento de 370 milhões anuais (EMBRAPA GADO DE LEITE, 2018). Essa produção tem elevada importância, principalmente no aspecto econômico, social e cultural visto que o queijo artesanal se destaca como a principal fonte de renda da região (ARCURI, 2018).

As queijarias credenciadas mineiras produzem queijos artesanais baseados em receitas passadas de geração em geração, caracterizadas pela utilização do fermento endógeno coletado da produção do dia anterior, o pingo (DORES *et al*., 2012; MARTINS, 2006).

Para a produção do queijo Canastra são conferidas diversas certificações, dentre elas, a de comercialização, concedidas pelo Instituto Mineiro de Agropecuária (IMA) e pelo Sistema Brasileiro de Inspeção de Produtos de Origem Animal (SISBI-POA).

Segundo o IMA 2017, os queijos minas artesanais (QMA) são assim categorizados como ***queijarias cadastradas*** caracterizada pelo comércio dentro do estado de Minas Gerais; ***queijarias relacionada*** vinculado a um entreposto de QMA; ***entreposto registrado*** conferido aos estabelecimentos que recebem, maturam e embalam os QMA, além da comercialização nacional pela inserção no SISBI-POA e as ***queijarias registradas***, elaboram o QMA com a maturação prevista na legislação realizando comércio nacional em razão da inserção no SISBI-POA.

Ao produtor cadastrado no IMA conforme a Lei 20.549, de 18 de dezembro de 2012, lhe é conferido o direito de comercializar QMA. O cadastro é requerido pelo *Sistema de Inspeção Municipal* (SIM) ou pela unidade mais próxima do IMA, assumindo a responsabilidade pela qualidade do queijo produzido ou do produto.

A Empresa de Assistência Técnica e Extensão Rural do Estado de Minas (EMATER) é responsável pelo auxílio ao produtor para que as exigências dos órgãos fiscalizadores sejam atendidas, a fim de garantir o cadastro no IMA. Essas exigências referem-se desde a sanidade do rebanho e aos equipamentos de produção até a infraestrutura das queijarias. Após o deferimento do cadastro, há uma inspeção contínua da produção dos queijos.

A outra certificação requerida para a comercialização do QMA, além do IMA, é a SISBI-POA, que faz parte do Sistema Unificado de Atenção a Sanidade Agropecuária (SUASA) e do Ministério de Agricultura Pecuária e Abastecimento (MAPA) regulamentado pela Lei nº 7.889/1989; inspeções desses sistemas são promovidas para garantir a segurança alimentar, além de demandar adequações na queijarias e ao tempo de maturação dos queijos (mínimo de 21 dias), o produtor que possuir o registro poderá comercializar o produto em todo o território nacional.

O intuito das certificações é assegurar a qualidade sanitária fornecendo produtos seguros para serem consumidos. Assim, há uma crescente demanda no mercado de queijos maturados devido a excelência no processamento e pela agregação de sabores diferenciados.

Dentre as principais regiões produtoras do QMA, a Região da Canastra caracteriza-se pela singularidade, unindo tradições e modernidade em um local privilegiado, o Parque Nacional da Serra da Canastra e a produção queijeira conquistaram o selo de indicação de procedência em 2011, pelo *Instituto Nacional da Propriedade Industrial* (INPI).

A APROCAN- *Associação dos Produtores de Queijo Canastra* é detentora da marca; além de buscar parcerias para a capacitação dos produtores da Região do Queijo Canastra, visa atender as demandas dos produtores quanto as certificações de acordo com a legislação. Esta associação é responsável por assegurar a qualidade da produção de seus filiados, sendo exigidos aos produtores associados que sigam as exigências contidas no Regulamento de Uso e marca coletiva. Neste regulamento constam visitas técnicas e monitoramento de análises laboratoriais para averiguar as propriedades físico-químicas e microbiológicas do produto submetendo-o à inspeções sensoriais para certificar se o mesmo atende as características descritas para o queijo Canastra, respeitando as normas de processamento envolvidas na cadeia produtiva de queijo (APROCAN, 2011).

A Canastra baseado na caracterização da EMATER-MG, foi reconhecida como produtora de queijo artesanal e posteriormente a microrregião foi caracterizada devido a produção tradicional de QMA, estabelecida pela Portaria do IMA nº. 694, de 17 de Novembro de 2004 (IMA, 2004).

O queijo da Serra da Canastra foi tombado pelo Instituto do Patrimônio Histórico e Artístico Nacional (IPHAN) como patrimônio cultural e imaterial brasileiro, a marca só é usada pelas propriedades dos sete municípios envolvidos, Araxá, Campo das Vertentes, Canastra, Cerrado, Serra do Salitre, Serro e Triângulo Mineiro, caracterizados pela marcante identidade cultural, conhecimento tradicional secular, tornando o produto artesanal e com aspectos singulares (IPHAN, 2008).

As bases originais dos queijos produzidos na região da Serra da Canastra foram provenientes da tradição portuguesa da Serra da Estrela, na região central de Portugal, que foram adornados pela identidade mineira transformando-se dinamicamente aderindo caracteres únicos ao longo do tempo. O queijo Canastra caracteriza-se pela qualidade e sabor inigualável, ganhadores do prêmio "Mondial du Fromage de Tours", na França, considerado como um dos mais renomados concursos de queijos mundial, sendo o primeiro produto brasileiro a conquistar medalha no torneio (EMATER,2017).

2.2 Aspectos intrínsecos, extrínsecos à produção e qualidade físico-química de queijos

O queijo é um concentrado lácteo que agrega uma fonte de vitaminas, fósforo, cálcio, sais minerais, lipídeos e carboidratos, além de ser considerado como um dos alimentos mais nutritivos, pela alta agregação de proteínas (PERRY, 2004).

Os parâmetros envolvidos na fabricação do queijo tais como, tratamento térmico associado ao tempo, processos de acidificação, maturação, técnicas de salga, temperos, ingredientes secundários, embalagens e condições na qual o leite é produzido, afetam diretamente a qualidade do produto final (GIELLO *et al*., 2017).

Os principais determinantes na aquisição, consumo e a preferência do consumidor por um determinado tipo de queijo está na peculiaridade dos produtos, nos aspectos sensitivos, sendo eles, a cor, o sabor e a textura.

Os aspectos sensoriais do queijo são influenciados pelo leite, que pode ser afetado pelos animais, pela nutrição dada ao gado, seja ela por feno ou silagem, a pastagem característica da região, somadas ao clima e as estações. Todos esses fatores contribuem para a geração de produtos diferenciados (YELURI JONNALA *et al*., 2018).

Dentre os componentes nutricionais fornecidos à vaca, as silagens de milho, por exemplo, apresentam uma maior concentração de amido disponível, formando mais ácidos graxos voláteis e, consequentemente, aumentando a produção de lactose, o teor de gordura e de proteína do leite (PHIPPS *et al.*, 2000). Desta forma, os padrões dos fotoperíodos, somadas as chuvas e a temperatura, são condições ambientais que impactam diretamente a fisiologia animal por conseguinte na produtividade leiteira (FERREIRA *et al*., 2015).

Queijos produzidos durante a primavera apresentam uma coloração mais amarelada do que os fabricados no inverno, sendo este fator influenciado pelo teor de caroteno presentes na forragem (W PARK; JEANJULIEN; SIDDIQUE, 2017). A cor dos queijos está também inerentemente associada ao teor de gordura do leite. Por isso, em função de todas essas variações, inclusive sazonais, muitas vezes são adicionados corantes ao queijo, como forma de uniformizar o produto (PERRY,2004).

A gordura do leite possui elevadas concentrações de ácidos graxos de cadeia curta, voláteis, os quais quando somadas à atividade proteolítica são responsáveis pela agregação do sabor e aroma de muitos produtos lácteos (W PARK; JEANJULIEN; SIDDIQUE, 2017). Nos queijos, esses ácidos contribuem para caracterizá-los em tipos distintos, gerando aromas que diferenciam os diversos tipos de queijo (SIMILI, LIMA; 2007).

Outro atributo organoléptico é a textura, influenciada pela atuação dos minerais durante a coagulação do leite, a qual também está intrinsicamente associada a retirada do soro. Quando removido em proporções relativamente pequenas, resulta em queijos moles. E, quando retirados em maiores quantidades, formam-se queijos mais duros (PERRY,2004).

Os variados métodos de fabricação de queijos possibilita que sejam acrescidos de características peculiares de cada região.

2.3 Processo produtivo do queijo

A produção de queijo consiste no processo de concentração do leite, podendo apresentar diferentes teores de gordura, composição nutricional e microbiota. Além da sua microbiota natural, o leite pode receber aporte de microrganismos presentes na superfície da teta, os quais podem ser encontrados também no queijo. Consequentemente, uma porcentagem significativa de espécies, dentre elas, *Brevibacterium linens, Staphylococcus equorum, e* bactérias do ácido lático *(BAL)* como, *Lactococcus lactis, Lactococcus chungangensis/raffinolactis e Lacticaseibacillus casei/paracasei*, envolvidas na produção de sabor, aroma e cor, podem contribuir para o metabolismo de proteínas e gorduras (FRÉTIN *et al* 2018; QUIGLEY *et al*., 2013).

A primeira etapa da produção consiste na coagulação da proteína do leite, a caseína, tornando-o mais espesso, constituindo um agregado de gorduras e sólidos, para a formação da massa do queijo.

Este dinamismo é propulsionado pela adição do fermento, no processamento tradicional do Queijo Canastra é utilizado o pingo, o qual é um fermento láctico natural, recolhido a partir do soro drenado do próprio queijo da produção do dia anterior. O uso do pingo transfere características específicas, somadas à atuação da microbiota autóctone do próprio leite, especialmente BAL, principais responsáveis pelas transformações que ocorrem durante a maturação. Essas bactérias atuam nos processos de lipólise e proteólise, produzindo compostos que propiciam ao produto textura e sabor, além de liberarem bacteriocinas, capazes de eliminar microrganismos patogênicos (MARTINS *et al.,* 2015).

Em Minas Gerais, os produtores de queijos da categoria Minas Artesanal, utiliza o pingo como fermento. Em países europeus são comercializadas as culturas iniciadoras, denominadas starters, que desempenham um papel crucial em todas as fases do processamento de queijos, principalmente durante a maturação. Essas culturas contém BAL mesofílicas e em algumas também estão presentes bactérias probióticas. À medida que essa microbiota cresce, converte a lactose em ácido lático que é intrinsicamente relacionada ao desenvolvimento de características organolépticas aceitáveis no produto alimentar (HØIER *et al*., 2010).

Formada a massa, esta será prensada retirando todo o excesso do soro. A forma que a massa é curada, propicia uma diversidade de queijos. Durante a etapa de maturação, a lise das

moléculas de proteínas e gorduras resultam em uma complexa combinação de substâncias que influenciam na textura, sabor e aroma do queijo. O desenvolvimento de sabor no queijo se deve à contribuição de leveduras, gerando um crescente interesse comercial na seleção de linhagens como agentes no processo de maturação (ROMANO *et al.*, 2006). Quanto maior o tempo de maturação, há uma maior intensificação do sabor e da consistência. Em tempo de maturação mais curto, o queijo apresenta sabor e consistência mais suave.

Quando envelhecidos, estes são dependentes de uma rica microbiota. Neste período ocorre uma série complexa de reações microbiológicas e bioquímicas, que estão associadas à temperatura, umidade e ao tempo de envelhecimento a qual este foi submetido, conferindo ao produto características peculiares (FOX; COGAN; GUINEE, 2017).

O processo de envelhecimento, consiste na formação de um biofilme na superfície do queijo, denominada casca, que contém uma variedade de espécies fúngicas e bacterianas, provenientes do leite cru e da adição de culturas iniciadoras introduzidas pelos queijeiros. A diversidade microbiana da casca evidencia os fatores que são capazes de influenciar na formação de comunidades resultantes das variações geográficas e ambientais (WOLFE *et al*., 2014; QUIGLEY *et al.*, 2013; FOX *et al*., 2004).

2.4 Leveduras do queijo

No processamento de alimentos e bebidas, as linhagens de leveduras utilizadas como entradas na fermentação são capazes de produzir diferentes metabólitos secundários, tais como álcoois superiores, acetatos, ácidos graxos e ésteres, que são responsáveis pela agregação do aroma e sabor (VARARU *et al*., 2016).

A ocorrência de leveduras em queijo é favorecida pela sua capacidade de crescer nas condições de baixas temperaturas de armazenamento, típicas dos ambientes de maturação, além de resistirem às altas concentrações de sal, baixo pH e atividade de água reduzida. Além disso, atuam em processos fermentativos da lactose e galactose e assimilação de succinato, ácidos láctico e cítrico (FRÖHLICH-WYDER; ARIAS-ROTH; JAKOB, 2019; FERREIRA; VILJOEN, 2003).

Estudos de Binetti *et al*. (2013), ao isolarem leveduras nativas presentes nos queijos argentinos, evidenciaram que dentre as espécies presentes, *Saccharomyces cerevisiae, Clavispora lusitaniae, Kluyveromyces lactis, Galactomyces geotrichum* e *Kluyveromyces marxianus* destacaram-se como as espécies predominantes. Todas as linhagens apresentaram boa resistência aos estresses do TGI, hidrofobicidade moderada e capacidade agregativa,

indicando a aplicabilidade que estas oferecem como probióticos ou até mesmo como culturas *starters* na produção de queijo. Outra vantagem descrita para justificar a utilização de *Kluyveromyces marxianus* como probiótico se caracteriza pelas propriedades antioxidantes que estas conferem para combater processos inflamatórios intestinais (CEUGNIEZ *et al*., 2017).

A crescente busca por potenciais leveduras probióticas nos queijos é crescente, dentre elas, *Debaryomyces hansenii* tem sido relatada devido à excelente capacidade de resistir às tensões do TGI, por sua forte adesão às células epiteliais originárias do cólon (Caco-2) induzindo à estimulação imunológica como produção de anticorpos, ativação de macrófagos e alterações na produção de algumas citocinas como Interleucina 10 (IL-10) atuando como uma citocina anti-inflamatória, e a Interleucina 12 (IL-12) presentes nas células mononucleadas do sangue periférico associadas às células de defesa T e Natural Killer (NK), quando comparada a linhagens de *Saccharomyces boulardii* já descritas na literatura como probióticas, apontando um maior efeito anti-inflamatório (OCHANGCO *et al*., 2016).

As leveduras isoladas dos queijos artesanais fabricados na Sérvia e na Croácia, quando submetidas a análises *in vitro* apresentaram-se como potenciais probióticas sendo a *K. lactis* altamente adesivas às células epiteliais originárias do colón (Caco-2) e *Torulaspora delbrueckii* é a mais resistente às condições quimicamente simuladas do TGI, caracterizando- as como potenciais imunomoduladoras (ZIVKOVIC *et al.*, 2014).

Kluyveromyces lactis são encontradas regularmente no leite e produtos lácteos, graças à sua capacidade de fermentação da lactose promovendo o seu crescimento no queijo (PADILLA *et al*., 2014). Estudos realizados no isolamento de leveduras do queijo Canastra, evidenciaram que *K. lactis* e a *T. delbrueckii* são leveduras promissoras para serem utilizadas na fermentação de substratos lácteos devido ao seu alto potencial na produção de voláteis aromáticos, podendo ser utilizadas na produção de queijos visando melhorias nas características sensoriais do produto final, evidenciando que são potenciais inoculantes em queijo (ANDRADE *et al*., 2019; ANDRADE *et al*., 2017).

Desta forma, as leveduras são potencialmente promissoras para a indústria alimentícia, a fim de obterem alimentos funcionais.

2.5 Microrganismos Probióticos

Probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, regularmente, conferem um benefício à saúde do hospedeiro (HILL *et al*., 2014). Caracterizam-se por contribuírem para a manutenção do equilíbrio da microbiota intestinal,

atuando positivamente diante às perturbações, dentre elas, intolerância à lactose, infecções no intestino e constipação (FULLER, 1989).

A potencialidade de um probiótico é alcançada, caso este não cause efeitos adversos ou patogênicos a mucosa, os quais quando avaliados devem possuir uma atividade de gelatinase, DNase e atividade hemolítica negativa. A Agência Nacional de Vigilância Sanitária (ANVISA, 2019) estabelece para um produto probiótico uma quantidade mínima viável de cultura entre 10^8 e 10^9 UFC por porção do produto. Para que seja considerado seguro e comercializado pelas indústrias alimentícias, são submetidos a processos de avaliação de segurança *in vitro* e *in vivo*, analisando seus aspectos funcionais (HILL *et al*., 2014).

É essencial que o probiótico seja capaz de suportar as barreiras naturais do hospedeiro, como acidez, ácidos graxos, sais biliares desconjugados a competição pelo local de adesão e nutrição entre as bactérias, resistindo à passagem pelo TGI e atuando beneficamente no intestino, sendo capaz de integrar temporariamente à microbiota local (SHEHATA *et al*., 2016; TYAGI, PRASAD, 2015; CZERUCKA *et al* 2007; NIELSEN, 1994; FULLER, 1989).

Desta forma, o leite e os produtos lácteos fermentados têm sido extensivamente estudados como alimentos funcionais, por apresentarem benefícios adicionais à qualidade de vida e conferir uma melhora na saúde, podendo reduzir os riscos de doenças. Isso se deve ao enriquecimento dos alimentos com microrganismos probióticos (GRANATO *et al*., 2010). Estudos elucidaram que microrganismos probióticos atuam como adjuvantes na prevenção primária de infecções, como a infecção por *Clostridioides difficile* (CDI) caracterizado como um dos patógenos entéricos mais letais nos Estados Unidos (MILLS; RAO; YOUNG, 2018).

Dentre as espécies probióticas que já estão bem estabelecidas no mercado e aplicadas pelas indústrias alimentícias são os *Lactobacillus* e *Bifidobacterium*. Dentre elas destacam-se *Lacticaseibacillus casei/paracasei* e *Lactococcus chungangensis/raffinolactis* (ITSARANUWAT *et al*., 2003), *Lactobacillus plantarum* (KLEEREBEZEM *et al*., 1997), *Bifidobacterium bifidum* (SCHIFFRIN *et al*., 1995) e *Bifidobacterium lactis* (CHIANG *et al*., 2000).

Atualmente, tem sido elucidado que bactérias probióticas não estão restritas apenas ao lúmen intestinal, podendo influenciar nos mecanismos de proteção da superfície de mucosa locais e a modulação de funções efetoras na imunidade adaptativa (CLANCY, 2003).

Dentre a diversidade microbiana existente, as leveduras possuem características que as tornam potenciais candidatas probióticas devido a tolerância aos fatores químicos e físicos, podendo resistir as tensões locais do trato gastrointestinal, como a presença de enzimas, sais

biliares, ácidos orgânicos, pH, além de possuir atividade antioxidante e antitumoral (SCHNURER *et al*., 2002).

A única levedura referida como probiótico para uso em humanos é a *Saccharomyces cerevisiae var. boulardii* (MORÉ; SWIDSINSKI, 2015; CZERUCKA *et al* 2007), embora tenha sido elucidada potenciais leveduras probióticas nos últimos anos, tais como *Pichia membranaefaciens* e *Candida oleophila*. Essas leveduras presentes em azeitonas, demonstraram capacidade para produzir metabólitos principais como na síntese de todas as vitaminas do complexo B e exibem atividades antibacterianas contra patógenos comuns de origem alimentar (SILVA *et al.*, 2011).

Em queijo se destaca as seguintes leveduras como potenciais probióticas, *Debaryomyces hansenii* (OCHANGCO *et al*., 2016; PADILLA *et al*., 2014), *K. lactis* (ANDRADE *et al*., 2019; FADDA *et al*., 2017; ANDRADE *et al.*, 2017; PADILLA *et al*., 2014; BINETTI *et al*., 2013), *K. marxianus* (FADDA *et al*., 2017; DIOSMA *et al*., 2014; PADILLA *et al*., 2014; BINETTI *et al*., 2013) e *T. delbrueckii* (ANDRADE *et al*., 2019; ANDRADE *et al*., 2017; ZIVKOVIC *et al.*, 2014).

CONSIDERAÇÕES GERAIS

No presente trabalho, as leveduras *K. lactis* B10 e *T. delbrueckii* B14 provenientes do isolamento do queijo Canastra foram utilizadas como culturas iniciadoras na produção de queijos. Essas leveduras contribuíram para o processo de maturação dos queijos agregando sabor e aroma. O estudo dessas leveduras demonstraram a potencialidade de serem utilizadas como culturas starters em produtos lácteos além de serem potenciais candidatas probióticas.

REFERÊNCIAS

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA- Probióticos Disponível em: <http://portal.anvisa.gov.br/resultado-de-busca?p_p_id=101&p_p_lifecycle=0&p_p_state=maximized&p_p_mode=view&p_p_col_id=column-1&p_p_col_count=1&_101_struts_action=%2Fasset_publisher%2Fview_content&_101_assetEntryId=2864062&_101_type=content&_101_groupId=219201&_101_urlTitle=probioticos&inheritRedirect=true> Acesso: 16 de Abril de 2019.

ANDRADE, R. P. *et al* Survival of *Kluyveromyces lactis* and *Torulaspora delbrueckii* to simulated gastrointestinal conditions and their use as single and mixed inoculum for cheese production. **Food Research International,** v. 125, p. 1- 12, 2019.

ANDRADE, R. P. *et al*. Yeasts from Canastra cheese production process: Isolation and evaluation of their potential for cheese whey fermentation. **Food Research International**, v. 91, p. 72–79, 2017.

APROCAN. Ata de Assembleia Geral Extraordinária da Associação dos Produtores de Queijo Canastra, 2011. Disponível em: <www.inpi.gov.br/menu-servicos/indicacao-geografica/arquivos/...de.../Canastra.pdf> Acesso; 11 de maio de 2019>.

ARCURI, PEDRO Pesquisa: desenvolvimento e inovação a serviço do leite- p.62-67. Disponível em: **ANUÁRIO LEITE 2018, EMBRAPA**. Edição Digital em embrapa.br/gado-de-leite. Acesso: 9 de Abril de 2019.

BINETTI, A. *et al*. Yeasts from autochthonal cheese starters: Technological and functional properties. **Journal of Applied Microbiology**, v. 115, n. 2, p. 434–444, 2013.

BRASIL, **Portaria no 146 de 7 de março de 1996.** Aprova o regulamento técnico de identidade e qualidade dos queijos. Diário Oficial da União, Brasília, 11 de março de 1996. Disponível em: <http://www.agais.com/normas/leite/queijos.htm> Acesso: 10 de Abril de 2019.

BRASIL. Instrução normativa n°.68. Método oficial para análise de alimentos de Origem animal. Ministério da Agricultura, Pecuária e Abastecimento. Diário Oficial da União Brasília, DF, 2017. Disponível em: <http://www.agricultura.gov.br/assuntos/laboratorios/legislacoes-e-metodos/poa/Manualdemtodosoficiaisparaanlisedealimentosdeorigemanimal2017.pdf> Acesso: 30 de junho de 2019.

BRASIL. Lei n°23157 de 18/12/2018 Norma Estadual- Minas Gerais- Dispõe sobre a produção e a comercialização dos queijos artesanais de Minas Gerais. Disponível em: < https://www.normasbrasil.com.br/norma/lei-23157-2018-mg_372516.html> Acesso: 09 de novembro de 2019.

CEUGNIEZ, A. *et al*. Interactions between Kluyveromyces marxianus from cheese origin and the intestinal symbiont Bacteroides thetaiotaomicron: Impressive antioxidative effects. **Lwt**, v. 81, p. 281–290, 2017.

CHIANG B. L.; SHEIH, Y. H.; WANG, L. H.; LIAO, C. K.; GILL, H. S. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium *(Bifidobacterium lactis HN019):* optimization and definition of cellular immune responses. **European Journal of Clinical Nutrition.** v. 54, p. 849–855, 2000

CLANCY R. Immunobiotics and the probiotic evolution. **FEMS Immunology Med Microbiology** 2003; 38: 9–12.

CZERUCKA, D; PICHE, T; RAMPAL, P. Review article: yeast as probiotics- Saccharomyces boulardii **Alimentary Pharmacology Therapeutics** 26, 767–778, 2007.

DIOSMA, G. *et al.* Yeasts from kefir grains: Isolation, identification, and probiotic characterization. **World Journal of Microbiology and Biotechnology**, v. 30, n. 1, p. 43–53, 2014.

DORES, M. T., & FERREIRA, C. L. L. F. Queijo minas artesanal, tradição centenária: ameaças e desafios. **Revista Brasileira de Agropecuária Sustentável (RBAS)**, v.2, n.2, p. 26-34. Dez 2012. Disponível em: <http://www.locus.ufv.br/handle/123456789/17724> Acesso em: 12 de Abril 2019.

EMBRAPA, 2018- Queijo minas artesanal agrega tradição cultural e valor para os produtores de leite Disponível em: <https://www.embrapa.br/milho-e-sorgo/busca-de-noticias/-/noticia/35193841/queijo-minas-artesanal-agrega-tradicao-cultural-e-valor-para-os-produtores-de-leite> Acesso: 09 de abril, 2019.

EMBRAPA GADO DE LEITE. LEITE oportunidades. p. 116, 2018.

EMATER, 2017- **Onze queijos de Minas foram premiados no Mondial du Fromage de Tours, na França. Disponível em:** <http://www.emater.mg.gov.br/portal.do?flagweb=novosite_pagina_interna&id=20986> Acesso: 09 de abril, 2019.

FADDA, M. E. *et al*. In vitro screening of Kluyveromyces strains isolated from Fiore Sardo cheese for potential use as probiotics. **LWT - Food Science and Technology**, v. 75, p. 100–106, 2017.

FERREIRA, G. A. *et al*. Estratégias de prevenção da mastite bovina no período de transição. **Veterinária em Foco**, v. 12, p. 80–91, 2015.

FERREIRA, A. D., & VILJOEN, B. C. Yeasts as adjunct starters in matured Cheddar cheese. International Journal of Food Microbiology, 86(1–2), 131–140, 2003.

FOX, P.F., MCSWEENEY, P.L.H., COGAN, T.M., AND GUINEE, T.P. (2004). Cheese: Chemistry, **Physics and Microbiology** Volume 1 (London: Academic Press).

FOX, P. F.; COGAN, T. M.; GUINEE, T. P. **Chapter 25 - Factors That Affect the Quality of Cheese**. Fourth Edition ed. [s.l.] Elsevier Ltd, 2017.

FULLER, A. A Review: Probiotics in man and animals. **Journal of Applied Bacteriology**, v. 66, p. 365-378, 1989.

FRÉTIN, M., MARTIN, B., RIFA, E., ISABELLE, V.-M., POMIÈS, D., FERLAY, A., et al. (2018). Bacterial community assembly from cow teat skin to ripened cheeses is influenced by grazing systems. **Science**. Rep. 8:200. DOI: 10.1038/s41598-017-18447-y

FRÖHLICH-WYDER, M. T.; ARIAS-ROTH, E.; JAKOB, E. Cheese yeasts. **Yeast**, v. 36, n. 3, p. 129–141, 2019.

GIELLO, M. *et al*. Dynamics of bacterial communities during manufacture and ripening of traditional Caciocavallo of Castelfranco cheese in relation to cows' feeding. **Food Microbiology**, v. 63, p. 170–177, 2017.

GRANATO, D. *et al*. Probiotic dairy products as functional foods. **Comprehensive Reviews in Food Science and Food Safety**, v. 9, n. 5, p. 455–470, 2010.

HILL, C. *et al.*, Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews Gastroenterology and Hematology**, August 2014, v. 11 p. 506-514.

HØIER, E. *et al*. The Production, Application, and Action of Lactic Cheese Starter Cultures. **Technology of Cheesemaking: Second Edition**, p. 166–192, 2010.

IMA- **PORTARIA Nº 694, DE 17 DE NOVEMBRO DE 2004**- Identifica a microrregião da Canastra. Disponível em: <www.ima.mg.gov.br › Material curso CFO/ CFOC> Acesso: 11 de maio de 2019>.

IMA- **Queijo Minas Artesanal, 2017**. Disponível em:<http://www.ima.mg.gov.br/queijo-minas-artesanal> Acesso: 11 de maio de 2019.

IMA- Dispõe sobre a produção e a comercialização dos queijos artesanais de Minas Gerais. **Lei nº 20549, de 18 de dezembro de 2012.** Disponível em: <www.ima.mg.gov.br> Acesso: 11 de maio de 2019>.

INPI- **Instituto Nacional de Propriedade Industria Queijo Canastra**, 2011. Disponível em:<www.inpi.gov.br/menu-servicos/indicacao-geografica/arquivos/...de.../Canastra.pdf> Acesso: 11 de maio de 2019.

IPHAN, **Instituto do Patrimônio Histórico e Artístico Nacional**, *Modo Artesanal de Fazer Queijo de Minas*. Disponível em: <www.iphan.gov.br/> Acesso em: 11 de maio de 2019.

KLEEREBEZEM, M.; BEERTHUYZEN, M. M.; VAUGHAN, E. E.; DE VOS, W. M.; KUIPERS, O. P. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. **Applied Environmental Microbiology**. v. 63, p. 4581-4593, 1997.

ITSARANUWAT P, AL-HADDAD KSH, ROBINSON RK. The potential therapeutic benefits of consuming health-promoting fermented dairy products: a brief update. **International Journal Dairy Technology**. v. 56 n. 4 p. 203-210, 2003.

LUND, B.; ADMASSON, I.; EDLUND, C. Gastrointestinal transit survival of an Enterococcus faecium probiotic strain administered with or without vancomycin. **International Journal Food Microbiology**. v. 77, p. 109-115, 2002.

MARTINS, J. M. Características físico-químicas e microbiológicas durante a maturação do queijo minas artesanal da região do Serro. **Tese – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos da Universidade Federal de Viçosa**. 2006. Disponível em: <http://www.locus.ufv.br/handle/123456789/499>. Acesso em: 12 de Abril de 2019.

MARTINS, J. M., GALINARI, É., PIMENTEL-FILHO, N. J., RIBEIRO JR, J. I., FURTADO, M. M., & FERREIRA, C. L. Determining the minimum ripening time of artisanal minas cheese, a traditional Brazilian cheese. **Brazilian Journal of Microbiology**, v.46, n.1, p. 219-230. 2015. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822015000100219> Acesso: 12 de Abril de 2019.

MATHARA, J. M.; SCHILLINGER, U.; KUTIMA, P. M.; MBUGUA, S. K.; GUIGAS, C.; FRANZ, C.; HOLZAPFEL, W. H. Functional Properties of Lactobacillus plantarum Strains Isolated from Maasai Traditional Fermented Milk Products in Kenya. **Current Microbiology**, v. 56, p. 315-321, 2008.

MILLS, J. P.; RAO, K.; YOUNG, V. B. Probiotics for prevention of Clostridium difficile infection. **Current Opinion in Gastroenterology**, v. 34, n. 1, p. 3–10, 2018.

MORÉ, M. I.; SWIDSINSKI, A. Saccharomyces boulardii CNCM I-745 supports regeneration of the

intestinal microbiota after diarrheic dysbiosis - A review. **Clinical and Experimental Gastroenterology**, v. 8, p. 237–255, 2015.

NIELSEN, E. M.; SCHLUNDT, J.; GUNVIG, A.; JACOBSEN, B. L. Epithelial, Mucus and Lumen Subpopulations of *Escherichia coli* in the Large Intestine of Conventional and Gnotobiotic Rats. **Microbial ecology in health and disease,** v. 7, p. 263-273, 1994.

OCHANGCO, H. S. *et al*. In vitro investigation of Debaryomyces hansenii strains for potential probiotic properties. **World Journal of Microbiology and Biotechnology**, v. 32, n. 9, p. 1–13, 2016.

PADILLA, B. et al. Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses. **International Dairy Journal**, v. 35, n. 2, p. 122–129, 2014.

PAULA, J. C. J; CARVALHO, A. F; FURTADO, M. M Princípios básicos de fabricação de queijo: do histórico à salga **Rev. Inst. Laticínios**. "Cândido Tostes", Mar/Jun, nº 367/368, 64: 19-25, 2009.

PERRY, K. S. P. Queijos: Aspectos Químicos, Bioquímicos E Microbiológicos. **Quimica Nova**, v. 27, n. 2, p. 293–300, 2004.

PHIPPS, R. H. *et al*. The effect of crop maturity on the nutritional value of maize silage for lactating dairy cows 3. Food intake and milk production. **Animal Science**, v. 71, n. 2, p. 401–409, 2000.

QUIGLEY, L., O'SULLIVAN, O., STANTON, C., BERESFORD, T.P., ROSS, R.P., FITZGERALD, G.F., AND COTTER, P.D. (2013). The complex microbiota of raw milk. **FEMS Microbiology**. Rev. 37, 664–698.

ROMANO, P., CAPACE, A., JESPERSEN, L. (2006). Taxonomic and ecological diversity of food and beverage yeasts. In A. Querol, & G. H. Fleet (Eds.), The yeast handbook e Yeasts in food and beverages (pp. 13e53). Heidelberg, germany: **Springer**-Verlag Berlin.

ROWLAND, I et al. "Current level of consensus on probiotic science--report of an expert meeting--London, 23 November 2009." **Gut microbes** v. 16, p. 436-439, 2010.

SCHIFFRIN, E.; ROCHAT, F.; LINK-AMSTER, H.; AESCHLIMANN, J.; DONNET-HUGHES, A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. **Journal Dairy Science** v. 78, p. 491-497, 1995.

SCHNURER, J. *et al*. Physiological characteristics of the biocontrol yeast J121. **FEMS Yeast Research**, v. 2, 2002.

SHEHATA, M. G., EL SOHAIMY, S. A., EL-SAHN, M. A., YOUSSEF, M. M. Screening of isolated potential probiotic lactic acid bacteria for cholesterol-lowering property and bile salt hydrolase activity. **Annals of Agricultural Sciences,** v. 61, p. 65–75, 2016.

SIMILI, F. F; LIMA, M. L. P; Como os alimentos podem afetar a composição do leite das vacas. **Pesquisa & Tecnologia**, vol. 4, n.1 Jan-Jun 2007; ISSN 2316-5146. Disponível em: <http://www.aptaregional.sp.gov.br/> Acesso: 30 de Abril de 2019.

SILVA, T. *et al*. Characterization of yeasts from Portuguese brined olives, with a focus on their potentially probiotic behavior. **LWT - Food Science and Technology**, v. 44, n. 6, p. 1349–1354, 2011.

TYAGI, A. K., PRASAD, S. (2015). Commentary: probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. **Frontiers in Microbiology**. v. 5, article 766, 2015.

VARARU, F. *et al.* Selection of aroma compounds for the differentiation of wines obtained by fermenting musts with starter cultures of commercial yeast strains. **Food Chemistry**, v. 197, p. 373–381, 2016.

W PARK, Y.; JEANJULIEN, C.; SIDDIQUE, A. Factors Affecting Sensory Quality of Goat Milk Cheeses: A Review. **Advances in Dairy Research**, v. 05, n. 03, 2017.

WOLFE, B. E. *et al*. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. **Cell**, v. 158, n. 2, p. 422–433, 2014.

YELURI JONNALA, B. R. *et al*. Sequencing of the Cheese Microbiome and Its Relevance to Industry. **Frontiers in Microbiology**, v. 9, n. May, p. 1020, 2018.

ZIVKOVIC, M. *et al.* Evaluation of the probiotic potential of yeasts isolated from traditional cheeses manufactured in Serbia and Croatia. **Journal of Intercultural Ethnopharmacology**, v. 4, n. 1, p. 12, 2014.

SEGUNDA PARTE- ARTIGOS*

ARTIGO 1- Evaluation *Kluyveromyces lactis* and *Torulaspora delbrueckii* as starter cultures in cheese and *in vitro and in vivo* characterization of their probiotic potential

ABSTRACT

Cheese, one of the most popular dairy foods in the world, presents remarkable diversity and organoleptic richness favored by microbial communities. Different yeasts have already been reported as relevant contributors to cheese characteristics. Besides, yeast in cheese has been evaluated in several studies regarding its probiotic potential. Recently we have shown that *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14, as mixed inoculum, positively impact the aroma of cheese, in addition to having probiotic potential. Thus, in this study, the main aims were to obtain a better characterization of the yeast's probiotic potential and also to evaluate their impact on cheese when inoculated in the mass or on the surface. These two yeast strains were subjected to co-aggregation with pathogens, survival in the intestinal environment and their protective effect against Salmonella infection. The *in vitro* safety assessment was performed using hemolysis and mucin tests. Recent studies have shown that the use of yeasts as starters cultures adds pleasant organoleptic aspects during maturation. The two yeast species were analyzed using two treatments inserting them into the surface and mass of the cheese. The physical-chemical properties were monitored for forty days to assess their impacts on the textural and sensory characteristics of the product. The results obtained showed that *K. lactis* B10 and *T. delbrueckii* B14 are interesting yeasts in the context of probiotics due to the absence of hemolytic activity and mucin degradation being considered a safety prerequisite for the selection of a probiotic strain. The *in vivo* conditions resulted in high survival through mice gastrointestinal tract. Also, the yeast *K. lactis* B10 when administered to mice resulted in a higher survival rate, after induction of the pathology with the pathogenic bacterium Salmonella. The yeasts remained viable throughout the process, with populations of 10^7 CFU/g for cheese with inoculation in the mass and 10^8 CFU/g for inoculation on the surface of the cheese. Yeasts when added to cheeses contributed to the maturation. When inoculated into the mass, the textural parameters showed less hardness and chewability, emphasizing that these factors interfere with the acceptance of the product by the consumer, thus having better sensory acceptability.

Keywords: Probiotic yeast. Starter culture. Mixed inoculum. Cheese.

1. INTRODUCTION

In recent years, yeasts have been widely used in various types of cheese, adding characteristic flavor, appearance, consistency to the product, in addition to prolonging the shelf life due to the ability to inhibit the growth of undesirable microorganisms (HELMY et al., 2019; BERESFORD *et al*., 2001). These microorganisms accelerate the maturation process and improve the cheese's flavor (HELMY et al., 2019; RAI, JEYARAM, 2015; ALVAREZ-MARTIN *et al.*, 2008). During the maturation process, lipolytic yeasts produce pleasant aromatic compounds such as alcohols, methyl ketones, and lactones (KANDASAMY et al., 2018).

The yeasts present in cheese are commonly derived from the use of starter cultures, processing equipment, or brine (ANDRADE et al., 2017; BANJARA; SUHR; HALLEN-ADAMS, 2015; IRLINGER et al., 2015). The use of these microorganisms in cheese production is favored due to the ability to grow and resist high concentrations of salt, low pH, reduced water activity, and low temperatures, typical conditions of the maturation process (FRÖHLICH-WYDER; ARIAS-ROTH; JAKOB, 2019; HELMY, 2019). The species commonly reported in cheese fermentation are *Torulaspora delbrueckii* (Andrade et al., 2017), *Debaryomyces hansenii* (IRLINGER et al., 2015; FERREIRA, VILJOEN; 2003), *Yarrowia lipolytica* (CEUGNIEZ et al., 2017; FERREIRA, VILJOEN; 2003) and the genus *Kluyveromyces* (FADDA et al., 2017; ANDRADE et al., 2017; IRLINGER et al., 2015).

Andrade et al. (2019) highlighted that among the yeasts isolated from the Canastra cheese, *K. lactis* and *T. delbrueckii* are promising species to be used as starter cultures in cheese production, especially when in mixed culture impacting the production of cheese desirable aromatic volatiles. These authors also highlighted that these yeasts had characteristics that make them interesting candidates for probiotics.

Studies on the probiotic potential of yeasts and their application in cheese have shown that in addition to the impact on product quality, these microorganisms can be transmitted via the product, making cheese a candidate for functional food. In studies such as de Oliveira et al., (2019), the probiotic potential of *K. lactis* strains, previously isolated from Serra da Canastra cheese was demonstrated with these yeasts maintaining high viability after exposure to simulated gastric and duodenal juices as well as had high self-aggregation, hydrophobicity, and β-galactose.

Studies carried out on yeasts in cheese have elucidated that some genera such as *Saccharomyces, Clavispora, Kluyveromyces, Galactomyces, Debaryomyces* and *Torulaspora*

have characteristics that make them potential probiotic candidates due to gastrointestinal resistance, moderate hydrophobicity, and aggregative capacity (ANDRADE et al., 2019; FADDA et al., 2017; FAKRUDDIN; HOSSAIN; AHMED, 2017; OCHANGCO et al., 2016; BINETTI et al., 2013). Among these yeasts, *K. lactis* was highly adhesive to the epithelial cells originating from the colon (Caco-2) and *T. delbrueckii* was the most resistant to the simulated conditions of the gastrointestinal tract (ZIVKOVIC et al., 2014).

In this context, scientists have been looking for potential probiotic yeasts to control enteric infections, an attractive possibility, since the use of antibiotics induces an imbalance of the intestinal bacterial microbiota in addition to causing acute diarrhea when administered. In recent years, several clinical trials in mice have been developed with the administration of potential probiotics that can collaborate for the prevention and treatment of a variety of enteric diseases, among them caused by *Salmonella enterica* serovar Typhimurium (ST) being one of the main causes of diarrhea (Pontier-Bres et al., 2015; Castillo et al., 2012). Pontier-bres et al., (2015) elucidated that patients who received the placebo with the probiotic yeast *Saccharomyces boulardii* (Sb-B) demonstrated to have beneficial effects for the treatment of infectious diarrhea caused by strains of *Salmonella*, *Shigella*, and *E. coli*. According to Veisseire et al., (2020) when isolating raw milk cheese yeasts when tested against ST, the strains *Saccharomyces cerevisiae* 16, Sc16; *Debaryomyces hansenii* 25, Dh25 were able to protect against infection in addition to having better adhesion than the commercial probiotic *S. cerevisiae* subspecies *boulardii* (CNCM I-1079, Sb1079).

Yeasts have potential characteristics to be used as probiotics due to their ability to survive a low pH, the presence of bile since stomach acidity, and the concentration of bile salts are the first biological barriers to be overcome. Also, its use in co-cultures expresses a significant role in the sensory and functional properties of cheese maturation, due to the formation of aromatic compounds and other metabolic activities. Andrade et al. (2019) when characterizing isolates of these same species, elucidated that they are potential probiotics demonstrating high survival to gastrointestinal conditions and resistance to different concentrations of temperatures and NaCl.

The objective of the present study was to characterize the probiotic potential of the yeasts *K. lactis* B10 and *T. delbrueckii* B14 previously isolated from Canastra cheese obtained from the work of Andrade et al. (2017), through safety tests and to evaluate their impact when inoculated in the cheese mass and on your surface during 40 days of maturation.

2. MATERIALS AND METHODS

2.1 OBTAINING YEASTS

The yeasts *K. lactis* B10 and *T. delbrueckii* B14 were obtained from the work of Andrade et al. (2017) from their isolation from the Canastra cheese production process, stored in cryovials at -20°C, maintained in the Microbiology laboratory at the Federal University of Lavras.

2.2 SECURITY ASSESSMENT

2.2.1 Preparing microorganisms and growth conditions

K. lactis B10 and *T. delbrueckii* B14 were grown in YPD (2% peptone, 2% glucose, and 1% yeast extract) incubated at 37°C for 24 hours with constant agitation (150 rpm). The yeasts were concentrated to obtain 10^9 CFU/mL according to Martins et al. (2013). For the tests, the bacteria were grown in BHI (Brain Heart Infusion) broth incubated at 37°C for 18h. The pathogenic bacteria used for the test were *Streptococcus pyogenes* ATCC 19615, *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* ATCC 29313, *Escherichia coli* EIEC CDC EDL 1284, *Shigella flexneri* ATCC 12022, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028.

2.2.2 Hemolysis test

Hemolytic activity was evaluated on plates containing Tryptic Soy Agar (TSA, Difco) supplemented with 5% sheep blood. The yeasts were sown on the surface and incubated at 37°C for 48 hours. The hemolytic reaction is characterized in (α), (β) and (γ) according to Buxton (2005) Alpha hemolysis (α) is observed through the formation of green or brown halos around the colonies, indicating partial loss of red blood cells; Beta hemolysis (β) forms light halos around the colonies indicating total loss of red blood cells and the absence of this activity is called gamma hemolysis (γ). The reaction was read by placing the plates against the light to assess the formation of light halos around the colonies and compared with the *Streptococcus pyogenes* ATCC 19615 positive control for β-hemolysis (Damaceno et al., 2017).

2.2.3 Mucin degradation assay

The mucin degradation test was performed as described by Damaceno et al. (2017), with modifications in Yeast Nitrogen Base (YNB, Difco) agar supplemented with 0.5% Type II porcine stomach mucin (M2378, Sigma, St. Louis, MO, USA). After culturing the yeasts in YPD, 5 µL of each culture was added to the surface of the YNB medium supplemented with mucin. The plates were incubated at 37 ° C for 48 hours. For reading, the plates were stained

with 0.1% black starch in acetic acid (3.5 M) for 30 minutes. Then, washed with acetic acid (1.2 M) until the discoloration of possible halos around the spots. A spot containing *S.* Typhimurium ATCC 14028 was added to each plate as a positive control.

2.2.4 Co-aggregation assays with pathogens

After cultivating the microorganisms, *K. lactis* B10, *T. delbrueckii* B14, *L. monocytogenes* ATCC 15313, *S. aureus* ATCC 29313, *E. coli* EIEC CDC EDL 1284, *S. flexneri* ATCC 12022, and *S.* Typhimurium ATCC 14028 were centrifuged at 10,000 g for 10 minutes, homogenized and washed twice with PBS solution. For the test tubes containing the control of each microorganism, a 2 mL aliquot was separated, stirred for 2 minutes, and subjected to absorbance reading at 530 nm using a microplate spectrophotometer (Epoch, BioTek Instruments, Inc., Winooski, VT, USA). Before reading the absorbance, 1 mL of yeast was mixed with 1 mL of each pathogen for 2 minutes. To read the absorbance after 3 hours, 100 µL of the surface of each tube was removed. The following formula was used to obtain the results, in which Ax corresponds to the absorbance of the yeast control tube and Ay to that of the pathogen control, while (x + y) corresponds to the absorbance of the tube containing the yeast-pathogen mixture: % co-aggregation = $[(Ax + AY) / 2 - A (x + y)] / [(Ax + AY) / 2] x 100$ (Vinderola & Reinheimer, 2003).

2.2.5 Survival in the intestinal environment

For *in vivo* tests, conventional *Swiss* mice were obtained from the Animal Care Center (CEBIO) of the Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, Brazil. The animals were ordered, divided into groups, and kept in ventilated cages (Alesco®, Monte Mor, SP, Brazil) under controlled conditions of temperature (22 ± 1°C), humidity (60–80%), light (light- 12 hours dark) and nutrition. Water and a commercial autoclavable pelleted food (Nuvilab CR1, Nuvital®, Curitiba, PR, Brazil) were sterilized by steam and administered ad libitum (Porto et al., 2019). The experimental conduct was carried out by the ethical rules promulgated by the National Council for the Control of Animal Experimentation (CONCEA) for the use of animals in behavioral research. This study was approved under protocol number 224/2019 by the Ethics Committee on the Use of Animals (CEUA/UFMG).

For colonization experiments, the mice received a daily dose of 0.1 mL containing 9.0 log CFU/mL by oral gavage for 10 days. At the end of the administration, the recently collected feces were diluted 100 times in saline and homogenized. Serial dilutions were made and a 0.1 mL aliquot was spread on Sabouraud Dextrose agar (Difco) supplemented with 200 mg/L of chloramphenicol. The plates were incubated at 37°C for 48-72 hours for yeast counting

(Martins et al., 2005). A group of mice not treated with yeast was maintained as a control to assess the presence of yeasts in the animals' normal microbiota.

2.2.6 Salmonellosis challenge

The probiotic treatment was administered for 10 days before the pathology was induced by *S.* Typhimurium (ATCC 14028). The experimental mice received a daily dose of 0.1 mL containing 9.0 log CFU/mL by oral gavage. After infection, treatment was continued throughout the experimental period. The control group received only sterile water by oral gavage, following the same procedure as their experimental counterparts. The contaminated mice were inoculated with 0.1 mL of *S.* Typhimurium (ATCC 14028) containing 6.0 log UFC/mL intragastrically.

To evaluate the effects of the treatment with the yeast on the morbidity and mortality during an experimental bacterial challenge, 33 mice were divided into 3 groups (n = 11 in each group): (ST) mice receiving sterile saline by oral gavage and challenged with *S.* Typhimurium, (B10 + ST) mice treated by oral gavage with *K. lactis* B10 and challenged with *S.* Typhimurium, and (B14 + ST) mice treated by oral gavage with *T. delbrueckii* B14 and challenged with *S.* Typhimurium. During 38 days (10 days of yeast pretreatment before challenge and 28 days post-challenge) mice were analyzed for clinical signs, weight, and mortality induced by *S.* Typhimurium infection (Martins et al., 2011).

2.3 PREPARATION OF INOCULUM

The yeasts *K. lactis* B10 and *T. delbrueckii* B14 were reactivated separately in liquid YPD (1% yeast extract; 2% peptone; 2% glucose) medium at 28°C, for 24 hours. Subsequently, they were subjected to multiplication to obtain populations in concentrations close to 10^8 and 10^9 centrifuged at 7000 rpm, at 4°C for 7 minutes to be inoculated in the cheeses (ANDRADE et al., 2019).

2.4 CHEESE PRODUCTION

Ten cheese were produced with two different treatments using a mixed inoculum of the yeasts *K. lactis* B10 and *T. delbrueckii* B14 in the mass and on your surface, respectively. The milk used to make the cheeses was previously pasteurized at 65°C for 30 minutes, then cooled to 38°C by adding calcium chloride and commercial rennet (HA-LA® Chr. Hansen Brazil) at a concentration of 1 mL/L. After the curd was formed, the excess whey was removed, shaped, and drily salted, the first tank the yeasts were added at a concentration of 10^8 cells/mL to the milk, and in the second tank, the inoculum was inserted in the same concentrations after salting

(ANDRADE et al., 2019). The cheeses were placed in trays and kept in a cold chamber at 15°C and 85% relative humidity.

The cheeses produced were evaluated for 40 days. Every seven days, the microbiological and physical-chemical properties of the product were evaluated.

At the beginning of maturation, the cheeses were 120 mm in diameter × 110 mm in height and weighed an average of 500 *g*, being turned four times a week, to guarantee the uniformity of the product.

2.5 YEAST VIABILITY DURING THE CHEESE MATURATION PROCESS

According to the methodology described by Borelli et al., (2006), the yeasts *K. lactis* B10 and *T. delbrueckii* B14, were plated in YPD medium, incubated at 28 ° C for 48 h to obtain the number of colonies forming units (UFC).

During the 40 days of cheese maturation, every seven days (T7, T14, T21, T30, and T40) microbiological analyzes were carried out to determine the viability of the yeasts *K. lactis* B10 and *T. delbrueckii* B14 in the product. For each treatment, 10 g of cheese samples were removed, mixed with 90 ml of buffered peptide water (BPW), and homogenized for approximately 5 minutes.

For plating, 100 µl of the sample was removed and transferred to an Eppendorf tube containing 900 µl of peptonized water (0.1%). Serial decimal dilutions were performed and a 100 µL aliquot of the 10^{-5} and 10^{-6} dilutions in duplicate were spread with Drigalsky loop, using the surface plating technique. Petri dishes were incubated at 28°C for 48 hours. Subsequently, microbial counting was performed.

2.6 PHYSICAL AND CHEMICAL ANALYSIS

2.6.1 Texture Profile Analysis (TPA)

To analyze the texture profile, cubic samples of cheese with a 2 cm edge were used in a TA-XT2 texturometer (Stable Micro Systems Ltd., Surrey, UK), containing a 75 mm diameter aluminum cylindrical probe, at a distance sample corresponding to 10 mm and the comprehension time 5 seconds, with a reading speed of 5 mm/s, post-test speed: 2 mm/s; the textural parameters evaluated were hardness, adhesiveness, elasticity, cohesiveness, gumminess, chewability and resilience according to Guiné et al., (2016).

2.6.2 Electron Microscopy

The cheese samples from each treatment were collected at the 21st day of maturation, taking care to obtain the surface and interior regions of the product, approximately 1 cm in

length, then they were placed in a 1.5 mL Eppendorf with the prior identification with the modified Karnovsky Fixative - composed of 2.5% glutaraldehyde, 2.5% formaldehyde in 0.0 5M sodium cacodylate buffer, pH 7.2, 0.001M $CaCl_2$, and kept in the refrigerator for one week. According to Alves et al. (2012) methodology for sample preparation.

The samples were washed using sodium cacodylate buffer (0.05 M), and then post-fixed in 1.0% osmium tetroxide for 2 hours. Then the samples were washed three times in distilled water and dehydrated in an acetone gradient (25%, 50%, 75%, 90%, once, and 100% three times), remaining for about 10 minutes in each solution.

After dehydration, the materials were taken to the oven to replace acetone with CO_2 to complete drying. The specimens obtained were mounted on aluminum stubs with double-sided carbon tape on an aluminum foil film and covered with gold in the evaporator at Sputtering Balzers SCD 050.

After the gold bath, the samples were examined using a scanning electron microscope, model Leo Evo 40, and electron micrographs were extracted.

2.7 CHROMATOGRAPHIC ANALYSIS

2.7.1 Determination of sugars by HPLC

Sugars were quantified by high-performance liquid chromatography (HPLC), using a Shimadzu chromatograph (Shimadzu Corp, Japan) equipped with a refractive index detector (RID-10A) and DAD diode array detector (SPD-10 Ai). The column employed was Supelcogel 8H (Supelco, Bellefonte, PA, USA) (7.8 mm x 30 cm) at 30 ° C in isocratic mode. The mobile phase used a 0.005 M H2SO4 solution at a flow rate of 0.5 mL/min. The identification and quantification were performed using internal standardization between the pure injected standard and the retention times of each peak (ANDRADE et al., 2017; DUARTE et al., 2010).

2.8 SENSORY ANALYSIS

Sensory analyzes were performed according to the work of Jo et al., (2018) on the twentieth, thirtieth, and fortieth day of maturation of the cheeses of the rind and mass treatments with untrained tasters at the Federal University of Lavras.

Panel participants received a sample of each treatment for tasting, water, and milk candies were delivered for cleaning the palate. The attributes evaluated were texture, aroma, flavor, and appearance, as well as the purchase intention and the sample preferred by the taster.

The samples of each treatment were coded and evaluated using the hedonic scale classified from 1 to 9, ranging from extremely disliked (1) to extremely liked (9).

3. RESULTS AND DISCUSSION

3.1 SECURITY ASSESSMENT

3.1.1 Hemolysis and mucin

For the establishment of a probiotic microorganism, some criteria must be met. In Brazil, for a given probiotic food to be deliberated, it is necessary to follow Resolution RDC ANVISA No. 241/2018, which establishes the inspection and standardization of safety tests, *in vivo* and in vitro, which include the characterization of the microorganism, the profile antimicrobial resistance, hemolytic activity, among others, to ensure that the strain is safe for its intended use, considering the target population and the recommended conditions of use (BRAZIL, 2019).

The yeasts *K. lactis* B10 and *T. delbrueckii* B14 have the benefit of being isolated from raw milk cheeses commonly consumed (ANDRADE et al, 2017). According to ANVISA, evidence that characterizes the history of use of the product containing the microbial lineage/species can also contribute to its proof of safety. Safety based on a history of safe use implies proof of lineage consumption for generations, on a large scale, and by a group of people with genetic heterogeneity, with no record of adverse events (BRASIL, 2019).

In addition to historical evidence of consumption, it is essential to carry out tests that directly assess the action of the strains using generally considered safety criteria, such as the hemolytic and mucin degradation capacity evaluated in the present study.

The absence of hemolytic activity is considered a safety prerequisite for the selection of a probiotic strain (FAO / WHO, 2002), which was treated by both strains, with a negative result. Thus, the use of these yeasts as probiotic cultures does not pose a risk to the host.

Mucin degradation is considered a pathogenicity factor, since this mucus layer of the gastrointestinal tract provides a protective barrier for the underlying epithelium against pathogenic microorganisms, in addition to chemical, physical or enzymatic damage (DERRIEN et al., 2004). Both *K. lactis* B10 and *T. delbrueckii* B14 proved to be safe in this regard, being unable to degrade mucin.

3.1.2 Co-aggregation assays with pathogens

The co-aggregation properties of probiotic strains with pathogens, as well as their ability to replace pathogens, are important for the therapeutic manipulation of the intestinal microbiota. The adherence of probiotics to pathogens prevents adherence to the intestinal epithelium by occupying their binding sites, favoring elimination by peristaltic movements. Consequently, the

ability to aggregate and co-aggregate are desirable properties for probiotics in foods that promote health (COLLADO et al., 2008).

To evaluate the ability of the strains to co-aggregate, enteric pathogens were used: *S. aureus*, *Shigella*, *S.* Typhimurium, *E. coli*, and *L. monocytogenes*. The commercialized probiotic strain *S. boulardii* was used as a comparative parameter.

Both *K. lactis* B10 and *T. delbrueckii* B14 presented the best results against *S.* Typhimurium with a co-aggregation of 62.26 ± 0.01 and 61.25 ± 0.01, respectively, while the highest co-aggregation value presented by *S. boulardii* was 60.46 ± 0.01 about *E. coli* (Table 1).

Table 1. Determination of ability of strains *K. lactis*, *T. delbrueckii*, and *S. boulardii* to co-aggregate with pathogens.

	% Co-aggregation (μ ± SD)				
	Pathogens				
	S. aureus	***Shigella***	***S.*** Typhimurium	***E. coli***	***L. monocytogenes***
Yeast					
K. lactis	58.5 ± 0.01	53.23 ± 0.00	62.26 ± 0.01	38.85 ± 0.00	50.6 ± 0.02
T. delbrueckii	42.12 ± 0.28	44.57 ± 0.02	61.25 ± 0.01	57.74 ± 0.02	39.51 ± 0.12
S. boulardii	55.86 ± 0.01	54.91 ± 0.00	57.06 ± 0.09	60.46 ± 0.01	44.12 ± 0.32

The results presented are similar to those found in the literature for other yeasts of the genus *Kluyveromyces*, in addition to that presented by other species such as *Pichia kluyveri*, *Pichia kudriavzevii*, *Issatchenkia orientalis*, *Hanseniaspora opuntiae*, and *Wickerhamomyces anomalus* (LARA- HIDALGO et al., 2019; OGUNREMI et al., 2019; OGUNREMI et al., 2015, BINETTI et al., 2013).

It is worth mentioning that it is possible that probiotics do not offer complete protection against infections. However, they could help to fight pathogens and thus reduce the duration and severity of symptoms (POPOVA et al, 2012).

3.1.3 Survival in the intestinal environment

The first requirement to be met in the characterization of new probiotic strains is resistance to passage through the gastrointestinal tract, which presents different adversities to the survival of microorganisms such as acidity, the presence of lytic enzymes, and bile salts. Initially, it is essential to ensure that populations will reach viable numbers in the intestine, to enable colonization and possible beneficial effects.

The strains were administered continuously, for 10 days in a population of 10^9 CFU/mL, the assessment of survival was made by counting viable cells present in feces collected on the last day of administration. The probiotic yeast *S. boulardii* was used as a comparative parameter.

The yeast *K. lactis* B10 was able to survive the passage through the gastrointestinal tract of mice, maintaining its viability at 1.42 x 10^8 CFU/g, that is, there was no significant mortality about the population initially administered. *T. delbrueckii* B14 also showed a high survival rate with an average population of 6.97 x 10^7 CFU/g, which did not differ significantly from the survival of *K. lactis* and *S. boulardii*, the survival of the latter being 5.40 x 10^7 CFU/g of fecal content.

The results are compatible with those reported for other species of *Kluyveromyces* and *Torulaspora* with survival above 80% in vitro studies (MACCAFERRI et al, 2011; ŽIVKOVIĆ et al, 2015, FADDA et al 2017, MORADE et al 2018, OLIVEIRA et al, 2019).

According to Raibaund (1992), bacterial strains must reach a minimum population level of 10^7 CFU/g of intestinal content to affect the intestinal ecosystem. Thus, the high viability of the strains shows that both yeasts have the necessary resistance conditions for colonization and possible probiotic actions.

3.1.4 Salmonellosis challenge

To assess the protective effect of probiotics by infection with *S.* Typhimurium, the mice received a daily dose of 0.1 mL containing 9.0 log UFC/mL of the yeasts *T. delbrueckii* B14 and *K. lactis* B10 by oral gavage for 10 days before *S.* Typhimurium infection, the treatment being continued throughout the 28-day experimental phase.

Most people infected with Salmonella develop diarrhea, fever, and abdominal cramps, 12 to 72 hours after infection. The disease usually lasts 4 to 7 days, with a large part recovering without treatment. However, in patients who develop enteric fever, diarrhea can be so severe

that they need to be hospitalized. In such cases, *Salmonella* spreads from the intestine into the bloodstream and then to other parts of the body and can cause death (CASTILLO et al., 2012).

It can be seen from Figure 1 that experiment deaths started 16 days after the onset of infection with 10% mortality in the control group compared to 20% mortality in the group that received treatment with *T. delbrueckii* B14, on the other hand, on the second day, there were no deaths in the group treated with *T. delbrueckii* B14 while the mortality rate in the control group rose to 30%. Both the control group and the group treated with *T. delbrueckii* B14 showed a similar evolution in terms of deaths, but it was possible to verify that, from the 21st day of infection on, the death rates in the treated group remained 10% lower than in the group control until the end of the analysis, with final survival of 50% and 40% respectively.

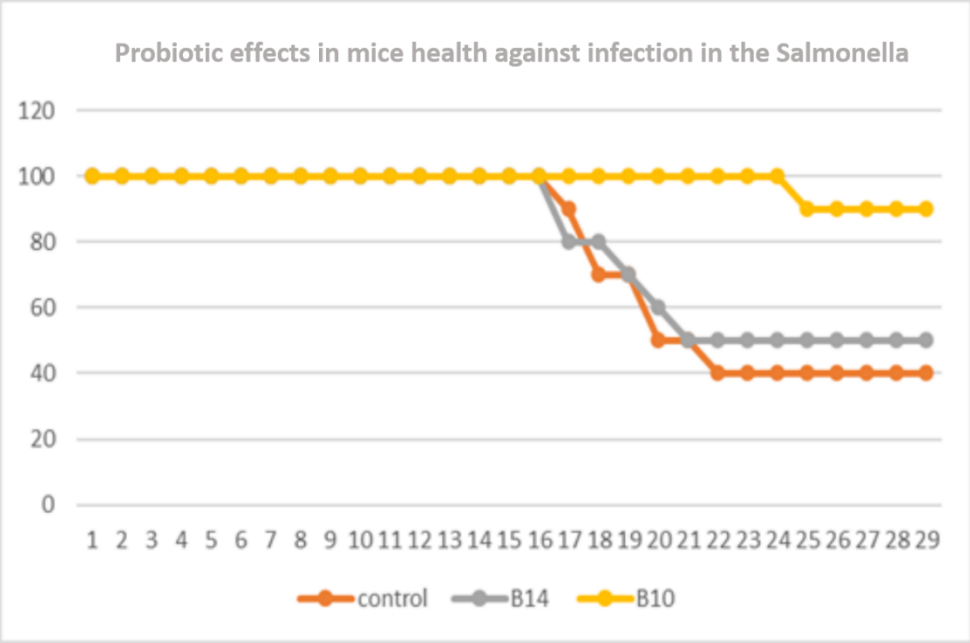


Figure 1. Probiotic effects in mice health against infection in the *Salmonella*. Control in orange - group without yeast administration. B14- Group in which *T. delbrueckii* B14 yeast was administered B10- Group in which *K. lactis* B10 yeast was administered.

While *T. delbrueckii* B14 had a slightly protective effect, the *K. lactis* B10 strain was able to maintain the survival of all test subjects up to 24 days after infection, with a mortality rate of only 10% that started on the 25th day and that remained until the end analysis.

The protective effect of *K. lactis* B10 was greater than the data found for probiotic bacteria. Asahara et al. (2011) in a study conducted with 8 different strains of *Lactobacillus* detected a protective effect of only 3 strains, which were able to confer a 62.5% survival in the tested test subject. Silva et al. (2004) using *Bifidobacterium longum* as a probiotic strain reported survival of only 40% of the test subject.

The probiotic yeast *Saccharomyces boulardii* has also shown a protective effect in infections caused by *S.* Typhimurium, reducing the inflammatory response and the invasion of these bacteria. Studies have shown that *S. boulardii* is capable of providing an average survival of up to 70% in infected mice (MARTINS et al., 2010, MARTINS et al., 2013, PONTIER-BRES et al., 2014; FRANCE et al., 2015). Other potential probiotic yeasts like *Saccharomyces cerevisiae* and *Pichia* also showed protective effects with survival rate in treated mice of 55% and 80% respectively, which shows that *K. lactis* B10 has extremely promising results (90% survival) about the other probiotics (MARTINS et al., 2005, FRANCE et al., 2015).

The mechanisms by which probiotics can protect against infections by pathogenic bacteria are multiple, variable, and dependent on each species.

The reduced level of mortality has generally been correlated with the protective effect of probiotics such as the reduction of systemic invasion of the pathogen, which generally causes extra-intestinal complications, including involvement of the central nervous system, cardiovascular system, pulmonary system, bones and joints, hepatobiliary system, among others (HUANG et al., 2005). In other words, the decrease in bacterial translocation can provide a decrease in systemic inflammation, a decrease in liver damage, and consequent lethality.

The data found in previous tests show a co-aggregation rate between *K. lactis* B10 and *S.* Typhimurium of 62%, elucidating that part of the protective effect is due to the link between yeast and the pathogen, which prevents bacterial translocation in addition to promoting the elimination of the pathogen in the stool.

3.2 MONITORING THE YEAST VIABILITY DURING THE CHEESE MATURATION PROCESS

Yeasts in both treatments remained viable throughout the maturation process. For the treatment of inoculation on the surface, the yeast populations remained stable throughout the maturation process. At 7 days of maturation a population of 2.15×10^8 CFU / g was found, at 14 days for 3.42×10^8 CFU / g, after 21 days, 2.03×10^8 CFU / g, at 30 and 40 days maturation, respectively 2.21×10^8 CFU / g and 1.25×10^8 CFU / g.

When yeast inoculation was carried out in the mass, the population on the 7th day was 2.88×10^7 CFU / g, 5.49×10^7 CFU / g at 14 days, and after 21 days 3.48×10^7 CFU / g. After 30 days of maturation, the populations was 1.60×10^8 CFU / g, while after 40 days of maturation, the population was 2.09×10^7 CFU / g.

In general, the inoculum on the cheese surface showed significantly higher populations about the treatment of yeasts in the mass, this is due to the distribution of yeasts in cheese as observed in electron micrographs (Figure 3). Yeasts remained viable throughout the days, demonstrating that they can be used as inoculants in the manufacture of cheese as described by Andrade et al. (2019) emphasizing that in the context of this work, the high survival of these yeasts are also a prerequisite to be commercialized as probiotic products.

During the maturation process, after seven days, the cheeses produced with the yeasts *K. lactis* B10 and *T. delbrueckii* B14 inoculated on the surface of the cheese showed less consistency as opposed to the treatment of yeasts inoculated in the mass that appeared more consistent (data contained in Table 2 of the Texture Profile Analysis topic). The data obtained agree with those reported by Tomar (2019) and González et al., (2020). According to these authors, the difference in textural parameters over time is intrinsically associated with metabolic and proteolytic activities and microbial distribution in cheese.

The cheeses produced with the yeasts inoculated on the surface showed greater hardness after the 14th maturation. This profile of greater hardness remained until the end of the maturation at 40 days (Table 2). In contrast to the inoculum in the mass that presented less hardness and formation of gaseous cavities resulting from the fermentation process (Figure 2 F-J) in agreement with the study by González et al. (2020) in which the cheeses also had gaseous cavities during maturation. Likewise, the results reported by Tomar (2019) were similar to those of the present study, with the cheese's hardness being increased during the ripening process.

As described by González et al. (2020) size, shape, appearance, and eye development distribution (the gas cavities) are some of the aspects that characterize and determine the quality of the cheese. It is observed that on the 40th day of cheese maturation with the inoculation of yeasts in the mass there is a fusion of the gas cavities (Figure 2 F-J). According to the Codex Alimentarius, the eye development distribution (the gas cavities) must have a regular shape and be well distributed.

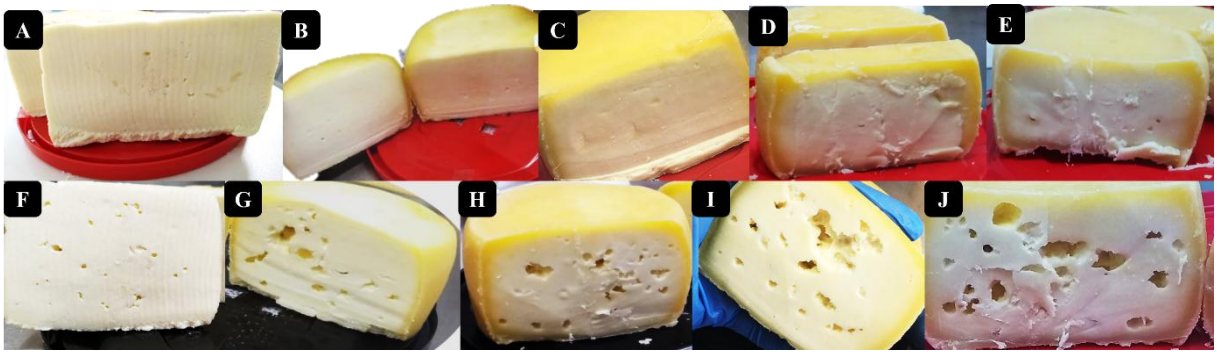


Figure 2 - Cheese profile after 40 days of maturation, yeasts *K. lactis* B10 and *T. delbrueckii* B14 inoculated on the surface of the cheese. In A: Time 7, B: Time 14, C: Time 21, D: Time 30, E: Time 40. Cheese profile at 40 days of maturation, yeasts *K. lactis* B10 and *T. delbrueckii* B14 inoculated in the mass of the cheese. In F: Time 7, G: Time 14, H: Time 21, I: Time 30, J: Time 40.

The formation of gas cavities in cheese may be associated with the assimilation of lactate, citrate, production of extracellular proteolytic and lipolytic enzymes together with the presence of CO_2, thus affecting the texture of the product (KHATTAB et al., 2019; MCSWEENEY; SOUSA, 2000; FERREIRA; VILJOEN, 2003). The yeasts present inside the cheese probably showed higher metabolic activity, forming more gas cavities when compared to the inoculation of the yeasts on the surface due to the larger area, distribution, and availability of sugar. As seen in the images generated by scanning electron microscopy (Figure 3) the distribution of yeasts inside the cheese was more uniform when they were inoculated into the mass.

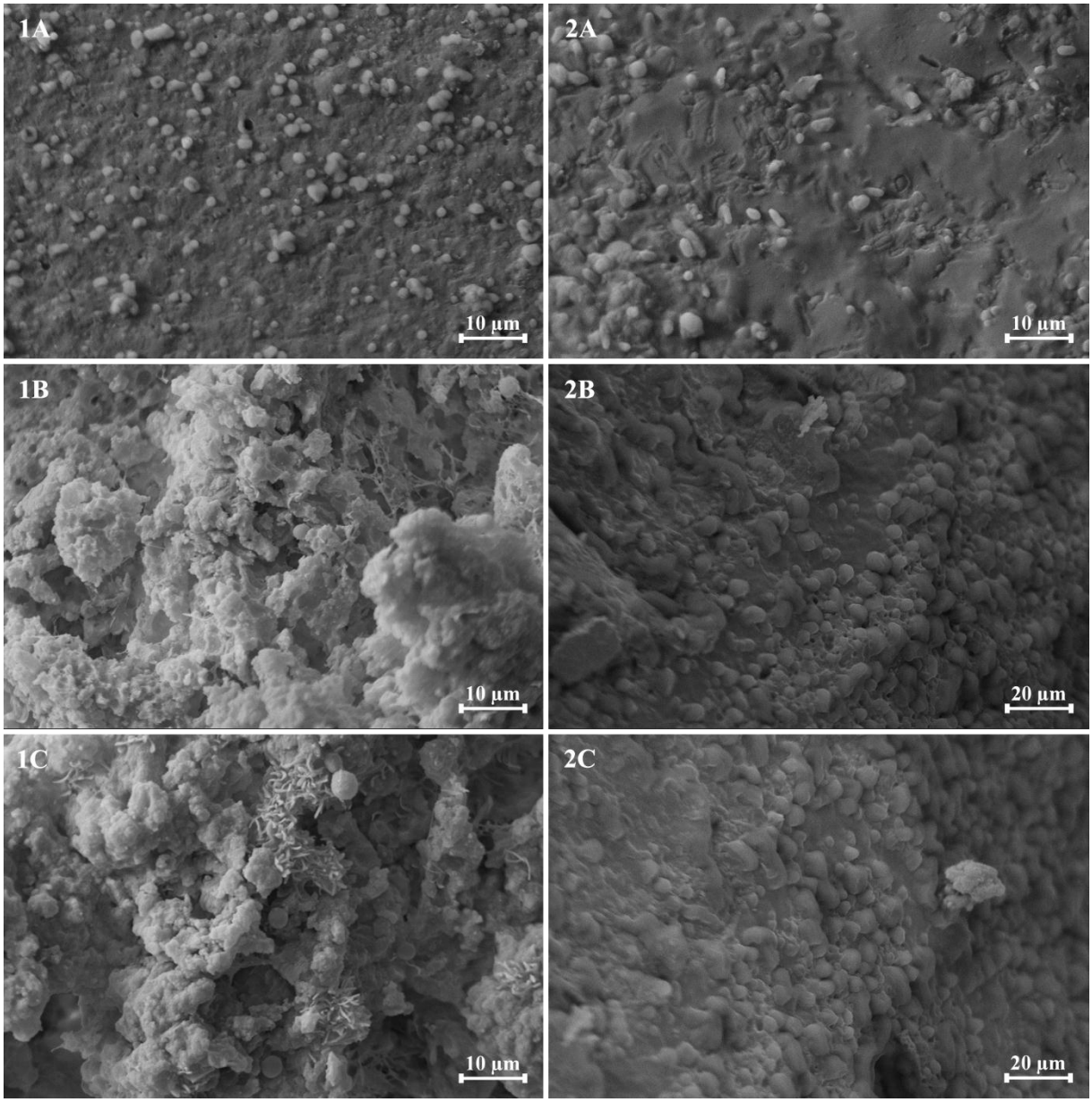


Figure 3 - *K. lactis* B10 and *T. delbrueckii* B14 yeasts present in cheese. 1A- Yeasts on the surface of the cheese in the inoculum of the rind at the 21st day of ripening, 2A- Yeasts on the surface of the cheese in the inoculum of the mass at the 21st day of maturation, 1B and 1C- Yeasts inside the cheese in the inoculum of the rind 21st day of maturation, 2B and 2C- Yeasts inside the cheese in the inoculum of the mass.

3.3 PHYSICAL AND CHEMICAL ANALYSIS

3.3.1 Texture Profile Analysis (TPA)

The textural properties of the cheeses were analyzed every seven days of the maturation period. The parameters analyzed were strength, hardness, adhesiveness, elasticity, cohesiveness, gumminess, chewability, and resilience.

The texturometer is analogous to human chewing by expressing the parameters mentioned above through the force-time curve. Through the mass, time, and distance, the deformation and disintegration of food are measured when a force is applied to it (KOHYAMA, 2020; KRAMER & SZCZENIAK, 1973). According to Childs & Drake (2009), texture is one of the main factors that influence the acceptability of cheeses and is inherently related to sensory appreciation (KOHYAMA, 2020; BOURNE, 1978).

The hardness, cohesiveness, elasticity, and adhesiveness are categorized as primary mechanical characteristics, the force required to compress food between the molars, generating a deformation in the product, is called hardness. Cohesiveness refers to the cheese's ability to deform until its internal structures break down. The rate obtained by the deformation of a material when it returns to its original condition when the deforming force is removed is defined as elasticity. Adhesiveness is the relationship between the forces of attraction between foods in contact with the mouth (KOHYAMA, 2020; FOX *et al*., 2017; SZCZESNIAK, 1963).

Secondary mechanical characteristics are fracturability, chewability, and gumminess. Fracturability is the ability of a material to fracture when a force is applied to it. Chewability makes the product suitable for ingestion; it is the product of the values obtained for hardness, cohesiveness, and elasticity. Gumminess, on the other hand, is the energy required for a product to be disintegrated to the point of being digested (KOHYAMA, 2020; FOX *et al.*, 2017; SZCZESNIAK, 1963).

The form of yeast inoculation and the maturation time affected the texture profiles as shown in table 2. After 7 days of maturation, the cheeses inoculated with the yeasts in the mass showed higher values of hardness (1147.66 N) and chewability (941.29 N), than the cheeses produced with the yeasts on the surface that presented, respectively, 755.21 N and 617.93 N (Table 2). These parameters over the maturation time increased so that the hardness and chewability in the inoculum on the surface showed higher values than the inoculum in the mass.

At 14 days of maturation, the cheeses with the inoculation on the surface showed a hardness of 1717.35 N while the chewability was 1302.91 N, whereas at that time in the inoculum in the mass the values were 2075.06 N and 1626.35 N, respectively (Table 2). After 21 days, the parameters analyzed for the rind were 1863.71 N and 1508.44 N, in contrast to the treatment of the mass that presented 1787.91 N and 1339.63 N (Table 2). After 30 days, the values expressed for the variables in the shell were 7334.11 N and 5541.14 N, for the mass 6969.60 N and 4596.07 N were obtained (Table 2). This relationship lasted until the end of the maturation process, where the hardness and chewability on the surface's cheeses showed values

of 7566.21 N and 5977.72 N while the cheeses in the mass showed 2783.14 N and 1797.51 N (Table 2), emphasizing that these factors interfere with the acceptance of the product by the consumer.

Among the parameters analyzed, the hardness increased during the maturation period, similar to the reports by Awad (2006). At the end of the maturation process, cheeses with yeast treatment on the surface showed higher hardness values as reported in the works by Jaster et al., (2019) about the treatment of yeast in the mass, this difference may be associated with changes in structural changes in the cheese matrix promoted by microbial metabolism according to its distribution in the product.

According to Desmasures et al., (2015) after the conversion of lactose into lactic acid, maturation in the matrix occurs in a synchronized manner, being responsible for altering the texture, flavor, and aroma of the cheese. Proteolysis and lipolysis of intracellular enzymes secreted by cell lysis contribute to the structure of the cheese matrix based on casein causing attenuation in the textural aspects making them softer as observed in the treatment of yeasts in the cheese mass.

During the maturation period, the increase in hardness was associated with moisture loss and, consequently, with an increase in salt concentration, resulting in structural changes in the cheese matrix, making it less elastic (FOX, et al. 2017; IRUDAYARAJ, et al., 1999). In this way, the elasticity over the maturation time was higher in cheeses with the treatment of yeasts on the surface, as described by Bockelmann (2011) the cheeses matured using a smear-ripened culture mix have an effect beneficial in the texture of the cheese contributing to a greater elasticity (Table 2).

Ercan et al. (2011) observed that the time of maturation, inoculation, and cheese processing can affect the values of cohesiveness. Thus, cohesiveness decreased over time in the treatment of yeast in the mass (Table 2).

The gumminess values of the cheeses increased during the maturation process, with the highest value in the treatment of yeasts in the skin with 6544.61 N while the yeasts when present in the mass presented 2034.60 N (Table 2). According to Rita et al. (2012), cohesiveness and gumminess do not significantly influence the textural properties of cheeses. The results obtained were similar to the studies by Carvalho et al. (2015) and Tomar (2019).

The data of the analysis of the texture profile of the cheeses produced were different, this is due to the processing of the same ones that were affected by the microbial distribution,

varying in the composition of acids and sugars, also evidenced in the works of Shan et al., (2019).

Table 2: Texture Profile Analysis (TPA) data for the treatment of yeasts on the cheese surface and cheese mass.

Days of Maturation/Inoculation										
	T7		T14		T21		T30		T40	
Attributes	**Surface**	**Mass**	**Surface**	**Mass**	**Surface**	**Mass**	**Surface**	**Mass**	**Surface**	**Mass**
Force	670.76±69.10	1080.02±152.69	1613.38±629.57	2047.81±60.42	1898.18±320.82	1678.82±91.93	7437.15±2692.57	6332.29±1499.26	7225.53±1850.14	2472.74±209.18
Hardness	755.21±36,51	1147.66±156.11	1717.35±676.07	2075.06±182.82	1863.71±359.91	1787.91±98.53	7334.11±3834.40	6969.60±1625.21	7566.21±1910.32	2783.14±229.29
Adhesiveness	-9,26±4.32	-1.96±0.18	-24.98±22.01	-2.88±4.52	-0.36±0.30	-82.89±70.73	-1.95±1.77	-26.47±36.56	-0.187±0.00	-0.14±0.13
Springiness	0.95±0.03	0.92±0.00	0.91±0.01	0.92±0.03	0.90±0.01	0.94±0.01	0.90±0.01	0.90±0.03	0.91±0.02	0.89±0.02
Cohesiveness	0.87±0.03	0.84±0.01	0.84±0.02	0.82±0.02	0.83±0.01	0.83±0.02	0.75±0.04	0.74±0.01	0.85±0.10	0.75±0.04
Gumminess	654.75±33.05	972.05±144.09	1437.38±534.62	1801.59±64.23	1668.62±265.61	1478.70±92.30	6163.06±2152.12	5209.11±1309.88	6544.61±990.86	2034.60±195.64
Chewiness	617.93±63.47	941.29±113.11	1302.91±487.88	1626.35±70.12	1508.44±225.70	1339.63±61.00	5541.14±1948.45	4596.07±1136.60	5977.72±850.89	1797.51±223.11
Resilience	0.52±0.01	0.51±0.01	0.47±0.04	0.47±0.01	0.47±0.03	0.47±0.03	0.37±0.04	0.38±0.02	0.35±0.17	0.41±0.03

SD*- Standard Deviation from Mean

3.4 CHROMATOGRAPHIC ANALYSIS

3.4.1 Determination of sugars by HPLC

The cheeses produced with mixed inoculum in the mass presented higher amounts of lactose in comparison to the mixed inoculum on the surface (Table 3). Andrade et al. (2019) observed that when yeasts, *K. lactis* B10 and *T. delbrueckii* B14 are used as mixed inoculum in the mass for cheese production, they can consume more than 90% of the present lactose; this is due to their uniform distribution in cheese. Throughout the maturation process, lactose was consumed more quickly in the inoculum in the mass compared to the inoculum on the surface.

Throughout the maturation process, yeasts inoculated in the mass were able to consume 53.08% of the present lactose, obtaining 14.79 g / Kg as the final product, whereas in the treatment that yeasts were present on the cheese surface, consumption was 5.44% dropping only 1.5 g / kg when compared to the beginning of the process.

Glucose in both treatments, suffered little oscillation over the maturation time with values below 1%, adding around 0.03 g / kg for cheeses with the initial inoculum on the surface from 11.38 g / kg to 11, 41 g / kg final and 0.08 g / kg for cheeses with the inoculum in the mass that initially contained 11.36 g / kg and 11.44 g / kg at the end.

Galactose maintained constant values for the inoculum in the mass, in contrast to cheeses with the inoculation of yeasts on the surface, which showed an increase of approximately 1% corresponding to 0.11 g / kg about the initial time corresponding to 11.28 g / kg obtaining 11.39 g / kg at the end.

Table 3: Concentration (g/kg) of carbohydrates cheese over 40 days of maturation.

Inoculation	**Time**	**Lactose**		**Glucose**		**Galactose**	
Mass	T7	31.52	±1.47	11.36	±0	11.23	±0
	T14	27.60	±3.80	11.41	±0.01	11.23	±0
	T21	30.39	±2.54	11.50	±0.01	11.26	±0
	T30	23.14	±3.61	11.50	±0.05	11.23	±0
	T40	14.79	±1.13	11.44	±0.035	11.23	±0
On the surface	T7	27.59	±0.32	11.38	0	11.28	±0.01
	T14	23.96	±3.06	11.41	±0.02	11.25	±0.01
	T21	31.41	±3.08	11.33	±0	11.34	±0.01
	T30	28.82	±1.92	11.33	±0	11.37	±0.01
	T40	26.09	±1.21	11.41	±0	11.39	±0.01

3.5 SENSORY ANALYSIS

The cheeses produced with the mixed inoculum in the mass and on the surface were subjected to three sensory evaluations at times 21, 30, and 40. The Principal Component Analysis (PCA) was performed using the Past 3.0 software (Oslo, Norway) the data were related to the texture, aroma, flavor, global appearance, and purchase intention factors for each treatment submitted through the weighted average obtained by the Sigma Plot 11.0 ® software.

The sensory analysis performed for the inoculum with the yeasts *K. lactis* B10 and *T. delbrueckii* B14 in the mass (Figure 4) demonstrated that the first two components PC1 (94.64%) and PC2 (5.36%) were responsible for 100 % of the variability in the same way as in the yeast mix on the surface (Figure 5), PC1 (57.65%) and PC2 (42.35%) explained 100% of the variability.

In both treatments, after 21 days of maturation, the cheeses produced showed more striking characteristics for the texture present in the upper right quadrant (positive side of PC1 and PC2) for the inoculum in the mass (Figure 4) and on the surface (Figure 5) in the quadrant lower right (positive side of PC1 and negative of PC2). Although the TPA values in both treatments (Texture Profile Analysis - Table 2) have declined in that time when compared to other textural analyzes.

At 30 days of maturation, the treatments showed more aroma and flavor than the other times (Figures 4 and 5) in the mass shown in the lower right quadrant (positive side of PC1 and negative side of PC2) and the rind in the upper right quadrant (positive side PC1 and PC2). After 40 days of maturation, there was no relationship with the analyzed parameters.

So that the appropriate maturation period for cheeses without compromising their organoleptic attributes would be 30 days since at 21 days there was an improvement in the texture of the product, but on the 30th day, there was a greater appreciation of the aroma and flavor by tasters, as the purchase intention was between 21 days and 30 days. Thus, it is concluded with these results of the sensorial analysis that the period of 40 days of maturation is not necessary.

In the first sensory analysis, the parameters analyzed were not significant ($p > 0.05$). Among the attributes understudy, people's perception of taste was greater for the treatment of yeasts in the mass, with an average of 7.13 than the treatment of yeasts in the skin, 5.41. The same occurs about the purchase intention, showing itself in favor of the treatment of the mass (Table 4).

The same pattern is observed in the second and third sensory analysis, with the taste of the cheese with the microorganisms inoculated in the mass showing greater averages of 7.07 and 6.41 about the cheeses with the microorganisms inoculated on the surface, presenting averages of 5.86 and 5.19. In this way, cheeses from the treatment of yeasts in the mass are tastier than cheeses made from yeasts on the surface (Table 4).

In all sensory analyzes, the estimate obtained for texture, aroma, and overall appearance among the intervals obtained with 95% confidence, the preference was the same for both treatments. Only the purchase intention and the taste of the mass treatment surpasses the rind treatment on average (Table 4).

IMA Ordinance No. 1736 of 07/27/2017 provides a minimum period of 22 days of maturation for cheeses from Serra da Canastra and establishes that it will be maintained until further research is carried out, ratifying or rectifying said maturation times. In this way, the present work conforms with the current legislation.

According to the report by Dores (2007) for Canastra cheese, it takes 22 days at room temperature (25 ° C) to reach safe microbiological standards, while at refrigeration temperature (10 ° C) 35 days. Martins et al., (2015) reported that after 17 days of maturation for artisanal cheese from Minas in the Serro region, it complied with the legislation, pointing out that this period is influenced by the season, regional styles, and temperatures. Campagnollo et al., (2018) demonstrated that the counting of the population of *L. monocytogenes* reduced by 4 logs UFC / g after the period of 15 to 21 days in cheeses produced with raw milk. After 22 days, the count of *L. monocytogenes* decreased to 5.8 log CFU / g, showing that the microbial diversity in raw milk added to the drop contributes to the reduction of *L. monocytogenes*.

According to Moskowitz and Krieger (1995) the three attributes that most influence the palatability of food are the taste, followed by the texture and appearance in agreement with the present work. According to Yates et al (2007), taste and texture are the main determinants for the acquisition of the product.

The use of yeast starter cultures contributed to the aggregation of flavor and texture during the maturation of some types of cheese due to their proteolytic and lipolytic activity leading to the shortening of the maturation process providing manufacturers with an economical approach in addition to contributing with consistent sensory aspects (PRICE et al., 2014; ROOSTITA; FLEET, 1996).

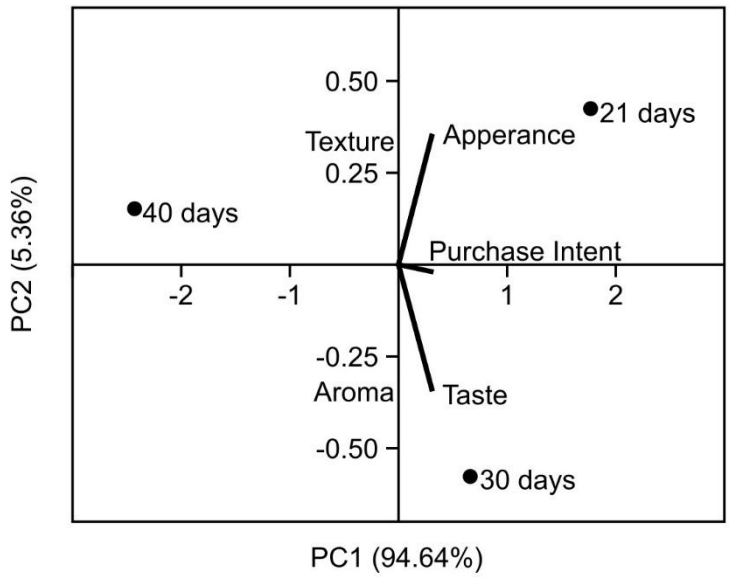


Figure 4 - Analysis of the main component (PCA) of sensory data of the inoculum in the mass vs days through the analysis of the weighted average related to the factors texture, aroma, taste, overall appearance, and purchase intention.

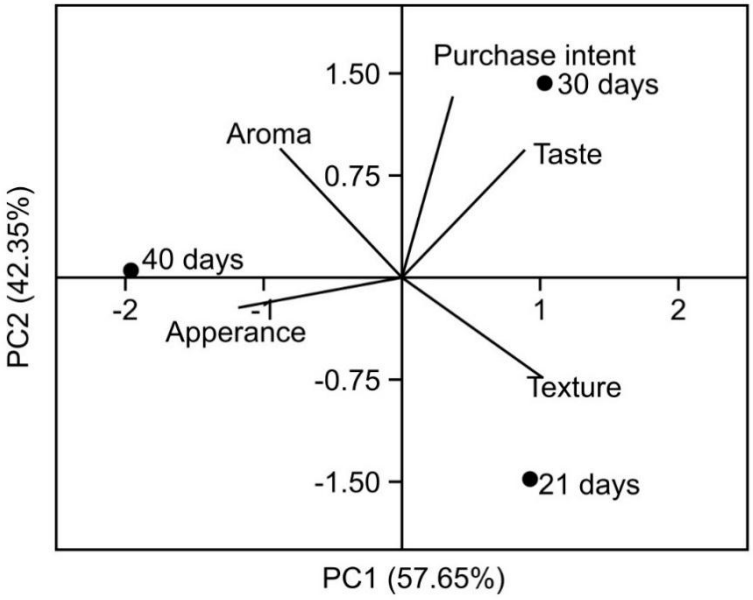


Figure 5 - Analysis of the main component (PCA) of sensory data of the inoculum on the surface vs days through the analysis of the weighted average related to the factors texture, aroma, taste, overall appearance, and purchase intention.

Table 4: Sensory analysis of cheeses produced with yeasts inoculated in the mass and on the surface at maturation times 21, 30, and 40 days.

Parameter	Inoculation on cheese mass Days of maturation			Inoculation on the cheese surface Days of maturation		
	21	30	40	21	30	40
Texture	7.72	7.63	7.47	7.31	7.21	7.06
Aroma	6.90	6.89	6.66	6.64	6.83	6.93
Taste	7.13	7.07	6.41	5.41	5.86	5.19
Appearance	7.67	7.57	7.51	7.25	7.24	7.66
Purchase intention	4.03	3.93	3.59	2.77	3.19	2.86

CONCLUSION

Cheese is a good alternative for the inoculation of probiotic microorganisms, as they confer some advantages among the others, such as low pH, in addition to offering a rich source of nutrients, reduced water activity, high content of lipids and proteins contributing to the cell viability.

K. lactis B10 and *T. delbrueckii* B14 remained viable during the 40 days of maturation. In this context, they are promising as a mixed inoculum in cheese production, in addition to providing the consumer market with a functional product, that is, safe and viable to be ingested as probiotics. In the tests carried out, qualitative aspects of the product were observed, such as pleasant taste, texture, and aroma.

REFERÊNCIAS

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA. Guia para instrução processual de petição de avaliação de probióticos para uso em alimentos. GUIA nº 21, versão 1, de 21 de fevereiro de 2019. P 13. Disponível em: http://portal.anvisa.gov.br/documents/10181/5280930/21.pdf/1c99eeb1-7143-469a-93ff-7b2b0f9187c0

ANDRADE, R. P. *et al*. Yeasts from Canastra cheese production process: Isolation and evaluation of their potential for cheese whey fermentation. **Food Research International**, v. 91, p. 72–79, 2017.

ANDRADE, R. P. *et al*. Survival of Kluyveromyces lactis and Torulaspora delbrueckii to simulated gastrointestinal conditions and their use as single and mixed inoculum for cheese production. **Food Research International**, v. 125, n. March, p. 108620, 2019.

ALVAREZ-MARTIN P, FLOREZ AB, HERNÁNDEZ-BARRANCO A, MAYO B. Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation. **Food Control** 19(1):62–70, 2008.

ALVES, E, PERINA, F.J, Apostila do Curso Introdutório a microscopia eletrônica de Varredura e Microanálise de Raio X. Editora UFLA, P. 25-29, 2012.

ASAHARA, T. et al. Efeito protetor da cepa *Shirota* de *Lactobacillus casei* contra infecção letal com *Salmonella enterica* serovar Typhimurium DT104 resistente a medicamentos em camundongos. **Jornal de Microbiologia Aplicada** , v. 110, n. 1, p. 163-173, 2011.

AWAD, S. Texture, and flavor development in Ras cheese made from raw and pasteurized milk. **Food Chemistry** v. 97, p. 394–400, 2006.

BANJARA, N.; SUHR, M. J.; HALLEN-ADAMS, H. E. Diversity of Yeast and Mold Species from a Variety of Cheese Types. **Current Microbiology**, v. 70, n. 6, p. 792–800, 2015.

BERESFORD, T.P; FITZSIMONS, N; BRENNAN, N. L; COGAN, T.M Recent advances in cheese microbiology. **International Dairy Journal** 11:259–274, 2001.

BINETTI, A. *et al*. Yeasts from autochthonal cheese starters: Technological and functional properties. **Journal of Applied Microbiology**, v. 115, n. 2, p. 434–444, 2013.

BOCKELMANN, W. Smear-Ripened Cheeses. **Cheese** p. -753-766, DOI: 10.1016/B978-0-12-374407-4.00089-3, 2011.

BORELLI, B. M. *et al*. Yeast populations associated with the artisanal cheese produced in the region of Serra da Canastra, Brazil. **World Journal of Microbiology and Biotechnology**, v. 22, n. 11, p. 1115–1119, 2006.

BOURNE, M. C. Texture profile analysis. **Food Technol.**, v. 32, n. 7, p. 62-66, 72, 1978.

BUXTON, R. Blood agar plates and hemolysis protocols. No1-9. Washington DC: **American Society for Microbiology**, 2005

CAMPAGNOLLO, F. B. et al. Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. **Food Microbiology**, v. 73, p. 288–297, 2018.

CARVALHO, B. S *et al.*, Physicochemical and rheological properties of Prato cheese during ripening. **African Journal of Biotechnology** v.14, p. 2028–2034, 2015.
CASTILLO, Natalia A. et al. Probiotics: an alternative strategy for combating salmonellosis: immune mechanisms involved. **Food Research International**, v. 45, n. 2, p. 831-841, 2012.

CEUGNIEZ, A. et al. Interactions between Kluyveromyces marxianus from cheese origin and the intestinal symbiont Bacteroides thetaiotaomicron: Impressive antioxidative effects. **Lwt**, v. 81, p. 281–290, 2017.

CHILDS, J. L., DRAKE, M. Consumer perception of fat reduction in cheese. **J. Sens. Stud. 24,** 902–921. 2009.

COLLADO, M. C., MERILUOTO, J., & SALMINEN, S. Adhesion and aggregation properties of probiotic and pathogen strains. **European food research and technology**, 226(5), 1065-1073. 2008.

DAMACENO, Q.S, SOUZA, J.P, NICOLI, J.R, PAULA, R.L, ASSIS, G.B, FIGUEIREDO, H.C, AZEVEDO, V, MARTINS, F.S. Evaluation of Potential Probiotics Isolated from Human Milk and Colostrum. **Probiotics Antimicrob Proteins**, 9(4):371-379, 2017. doi: https://doi.org/10.1007/s12602-017-9270-1.

DE MELO PEREIRA, G. V., DE OLIVEIRA COELHO, B., JÚNIOR, A. I. M., THOMAZ-SOCCOL, V., & SOCCOL, C. R. How to select a probiotic? A review and update of methods and criteria. **Biotechnology advances**, 36(8), 2060-2076, 2018.

DERRIEN, M., VAUGHAN, E. E., PLUGGE, C. M., & DE VOS, W. M. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. International journal of systematic and evolutionary microbiology, 54(5), 1469-1476. 200

DESMASURES, N et al. Smear Ripened Cheeses - **Diversity, dynamics, and functional role of actinomycetes on European smear-ripened cheeses**. v.1, p. 1-18, 2015.

DORES, Milene Therezinha das. Artisanal Minas cheese from the Canastra Mountain Range matured at room temperature and under refrigeration. 2007. 103 f. Dissertação (Mestrado em Ciência de Alimentos; Tecnologia de Alimentos; Engenharia de Alimentos) - Universidade Federal de Viçosa, Viçosa, 2007.

DUARTE, W. F. *et al.* Characterization of different fruit wines made from cacao, cupuassu, gabiroba, jaboticaba, and umbu. **LWT - Food Science and Technology**, v. 43, n. 10, p. 1564–1572, 2010.

ERCAN, D, et al. Physicochemical textural volatile and sensory profiles of traditional Sepet cheese. **Journal of Dairy Science** v. 94, p. 4300–4312, 2011.

FADDA, M. E. et al. In vitro screening of Kluyveromyces strains isolated from Fiore Sardo cheese for potential use as probiotics. **LWT - Food Science and Technology**, v. 75, p. 100–106, 2017.

FAKRUDDIN, M.; HOSSAIN, M. N.; AHMED, M. M. Antimicrobial and antioxidant activities of Saccharomyces cerevisiae IFST062013, a potential probiotic. **BMC Complementary and Alternative Medicine**, v. 17, n. 1, p. 1–11, 2017.

FAO/WHO. Guidelines for the Evaluation of Probiotics in Food. **Joint FAO/WHO** Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food, p. 1–11. 2002.

FERREIRA, A. D., & VILJOEN, B. C. Yeasts as adjunct starters in matured Cheddar cheese. **International Journal of Food Microbiology**, 86(1–2), 131–140, 2003.

FRANCE, R. C, CONCEIÇÃO, F. R, MENDONÇA, M. et al. Pichia pastoris X-33 possui propriedades probióticas com notável atividade antibacteriana contra Salmonella Typhimurium. **Appl Microbiol Biotechnol** 99, 7953-7961 (2015) <https://doi.org/10.1007/s00253-015-6696-9>.

FOX, P. F, GUINEE TP, COGAN, T.M, MCSWEENEY, P. L. H Cheese: Structure, Rheology, and Texture. In: **Fundamentals of Cheese Science**. Springer, Boston, MA. https://doi.org/10.1007/978-1-4899-7681-9,14 p. 475-532, 2017.

FRÖHLICH-WYDER, M. T.; ARIAS-ROTH, E.; JAKOB, E. Cheese yeasts. **Yeast**, v. 36, n. 3, p. 129–141, 2019.

GIL-RODRÍGUEZ, A M.; CARRASCOSA, A. V.; REQUENA, T. Yeasts in foods and beverages: in vitro characterization of probiotic traits. **LWT- Food Science and Technology**, v. 64, n. 2, p. 1156-1162, 2015.

GUINÉ, R. P. F. *et al.* Analysis of factors influencing the physical, chemical, and sensorial properties of Serra da Estrela cheeses. **Journal of Food Measurement and Characterization**, v. 10, n. 3, p. 643–657, 2016.

GONZÁLEZ, M. et al. Acoustic techniques to detect eye formation during ripening of Emmental type cheese. **Innovative Food Science and Emerging Technologies**, v. 59, n. November 2019, p. 102270, 2020.

HELMY, E. A. et al. Evaluation of potentially probiotic attributes of certain dairy yeast isolated from buffalo sweetened Karish cheese. **Heliyon**, v. 5, n. 5, p. e01649, 2019.

IRLINGER, F. et al. Cheese rind microbial communities: Diversity, composition, and origin. **FEMS Microbiology Letters**, v. 362, n. 2, 2015.

IRUDAYARAJ, J. et al. Texture development in cheddar cheese during ripening. **Canadian Biosystems Engineering / Le Genie des biosystems au Canada**, v. 41, n. 4, p. 253–258, 1999.

IMA **Portaria IMA Nº 1736 DE 27/07/2017.** Disponível em:< https://www.legisweb.com.br/legislacao/?id=346814> Acesso: 21 de Julho de 2020.

JASTER, H. et al. Quality assessment of the manufacture of new ripened soft cheese by Geotrichum candidum: Physico-chemical and technological properties. **Food Science and Technology**, v. 39, n. 1, p. 50–58, 2019.

JO, Y. et al. Sensory and chemical properties of Gouda cheese. **Journal of Dairy Science**, v. 101, n. 3, p. 1967–1989, 2018.

KANDASAMY, S.; KAVITAKE, D.; SHETTY, P. H. Lactic Acid Bacteria, and Yeasts as Starter Cultures for Fermented Foods and Their Role in Commercialization of Fermented Foods. **Innovations in Technologies for Fermented Food and Beverage Industries**, p. 25–52, 2018.

KOHYAMA, K. Food Texture – Sensory Evaluation and Instrumental Measurement. **Textural Characteristics of World Foods**, v. 1, n. Kohyama 2015, p. 1–13, 2020.

KRAMER & SZCZENIAK, A. S. **Texture measurements of foods.** Boston: D. Reidel. Publishing Company, p. 175, 1973.

KHATTAB, A. R. et al. Cheese ripening: A review on modern technologies towards flavor enhancement, process acceleration, and improved quality assessment. **Trends in Food Science and**

Technology, v. 88, n. March, p. 343–360, 2019.

KIRK, Martyn D. et al. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. **PLoS medicine**, v. 12, n. 12, 2015.

LARA-HIDALGO, C. E. et al. Isolation of Yeasts from Guajillo Pepper (Capsicum annuum L.) Fermentation and Study of Some Probiotic Characteristics. **Probiotics and antimicrobial proteins**, v. 11, n. 3, p. 748-764, 2019.

MACCAFERRI, S., KLINDER, A., BRIGIDI, P., CAVINA, P., & COSTABILE, A. Potential probiotic Kluyveromyces marxianus fragilis B0399 modulates the immune response in Caco-2 cells and PBMCs and impacts on the human gut microbiota in an in vitro colonic model system. **Applied and Environmental Microbiology**, AEM-06385. 2011.

MARTINS, F.S, NARDI, R.M, ARANTES, R.M, ROSA, C.A, NEVES, M.J, NICOLI, J.R. Screening of yeasts as probiotic based on capacities to colonize the gastrointestinal tract and to protect against enteropathogen challenge in mice. **The Journal of general and applied microbiology**, 51(2):83-92, 2005. DOI: https://doi.org/10.2323/jgam.51.83

MARTINS, F.S, ELIAN, S.D, VIEIRA, A.T, TIAGO, F.C, MARTINS, A.K, SILVA, F.C, SOUZA, E.L, SOUSA, L.P, ARAÚJO, H.R, PIMENTA, P.F, BONJARDIM, C.A, ARANTES, R.M, TEIXEIRA, M.M, NICOLI, J.R. Oral treatment with Saccharomyces cerevisiae strain UFMG 905 modulates immune responses and interferes with signal pathways involved in the activation of inflammation in a murine model of typhoid fever. **Int J Med Microbiol**, 301(4):359-364 2011. DOI: https://doi.org/10.1016/j.ijmm.2010.11.002.

MARTINS, F.S, VIEIRA, A.T, ELIAN, S.D, ARANTES, R.M, TIAGO, F.C, SOUSA, L.P, ARAÚJO, H.R, PIMENTA, P.F, BONJARDIM, C.A, NICOLI, J.R, TEIXEIRA, M.M. Inhibition of tissue inflammation and bacterial translocation as one of the protective mechanisms of Saccharomyces boulardii against Salmonella infection in mice. **Microbes Infect**, 15(4):270-279, 2013. DOI: https://doi.org/10.1016/j.micinf.2012.12.007

MARTINS, J. M. et al. Determining the minimum ripening time of artisanal Minas cheese, a traditional Brazilian cheese. **Braz. J. Microbiol.**, São Paulo, v. 46, n. 1, p. 219-230, Mar.2015. Disponível:<http://www.scielo.br/scielo.php?script=sci_arttext&pid=S151783822015000100219&lng=en&nrm=iso>. 21 July 2020. http://dx.doi.org/10.1590/S1517-838246120131003.

MARTINS, F. S., DALMASSO, G., ARANTES, R. M., DOYE, A., LEMICHEZ, E., LAGADEC, P., & CZERUCKA, D. (2010). Interaction of *Saccharomyces boulardii* with *Salmonella* enterica serovar Typhimurium protects mice and modifies T84 cell response to the infection. **PLoS One**, 5(1).

MATERA, J., LUNA, A. S., BATISTA, D. B., PIMENTEL, T. C., MORÃES, J., KAMIMURA, B. A., CRUZ, A. G. Brazilian cheeses: A survey covering physicochemical characteristics, mineral content, fatty acid profile, and volatile compounds. **Food Research International**, 108, 18–26, 2018.

MORADI, R., NOSRATI, R., ZARE, H., TAHMASEBI, T., SADERI, H., OWLIA, P. Screening and characterization of in-vitro probiotic criteria of Saccharomyces and Kluyveromyces strains. **Iranian Journal of Microbiology**, 10(2), 123. 2018.

MCSWEENEY, P. L. H., SOUSA, M. J. Biochemical pathways for the production of flavor compounds in cheeses during ripening: A review. **Lait** 80, p. 293–324, 2000.

MOSKOWITZ, H. R; KRIEGER, B. "The contribution of sensory liking to overall liking: An analysis of six food categories." **Food Quality and Preferen**ce. V.6 p. 83–90, 1995.

OCHANGCO, H. S. *et al*. In vitro investigation of Debaryomyces hansenii strains for potential probiotic properties. **World Journal of Microbiology and Biotechnology**, v. 32, n. 9, p. 1–13, 2016.

OGUNREMI, O. R.; SANNI, A. I.; AGRAWAL, R. Probiotic potentials of yeasts isolated from some cereal-based Nigerian traditional fermented food products. **Journal of applied microbiology**, v. 119, n. 3, p. 797-808, 2015.

OLIVEIRA, D. R. et al. Selection of potentially probiotic Kluyveromyces lactis for the fermentation of cheese whey-based beverage. **Annals of Microbiology**, v. 69, n. 13, p. 1361–1372, 2019.

PONTIER-BRES, R., MUNRO, P., BOYER, L., ANTY, R., IMBERT, V., TERCIOLO, C., ANDRÉ, F., RAMPAL, P., LEMICHEZ, E., PEYRON, J. F., & CZERUCKA, D. (2014). *Saccharomyces boulardii* modifies *Salmonella* Typhimurium traffic and host immune responses along the intestinal tract. **PloS one**, 9(8), e103069. https://doi.org/10.1371/journal.pone.0103069

POPOVA, M., MOLIMARD, P., COURAU, S., CROCIANI, J., DUFOUR, C., LE VACON, F., & CARTON, T. Efeitos benéficos dos probióticos nas infecções do trato respiratório superior e suas ações mecânicas para antagonizar patógenos. **Jornal de microbiologia aplicada** , 113 (6), 1305-1318. 2012.

PORTO, B.A.A, MONTEIRO, C.F, SOUZA, É.L.S, LEOCÁDIO, P.C.L, ALVAREZ-LEITE, J.I, GENEROSO, S.V, CARDOSO, V.N, ALMEIDA-LEITE, C.M, SANTOS, D.A, SANTOS, J.R.A, NICOLI, J.R, PESSIONE, E, MARTINS, F.S. Treatment with selenium-enriched Saccharomyces cerevisiae UFMG A-905 partially ameliorates mucositis induced by 5-fluorouracil in mice. **Cancer Chemother Pharmacol**, 84(1):117-126, 2019. doi: https://doi.org/10.1007/s00280-019-03865-8.

PRICE, E. J. et al. Study of the influence of yeast inoculum concentration (Yarrowia lipolytica and Kluyveromyces lactis) on blue cheese aroma development using microbiological models. **Food Chemistry**, v. 145, p. 464–472, 2014.

RAIBAUD, Pierre. Bacterial interactions in the gut. In: Probiotics . **Springer**, Dordrecht, 1992. p. 9-28.

RISHI, Praveen et al. Protective efficacy of probiotic alone or in conjunction with a prebiotic in Salmonella-induced liver damage. **FEMS Microbiology Ecology,** v. 69, n. 2, p. 222-230, 2009.

RITA, C. S. N; SILVA, R.C.S.N; MINIM, V. P. R; VIDIGAL, M. C. R T; SILVA, A; SIMIQUELI, A. A; MINIM, L. A. Sensory and instrumental consistency of processed cheeses. **Journal of Food Research** v. 1, p. 204–213, 2012.

ROOSTITA, R.; FLEET, G. H. The occurrence and growth of yeasts in Camembert and Blue-veined cheeses. **International Journal of Food Microbiology**, v. 28, n. 3, p. 393–404, 1996.

SHAN, J. et al. Characterization of the Processing Conditions upon Textural Profile Analysis (TPA) Parameters of Processed Cheese Using Near-Infrared Hyperspectral Imaging. **Analytical Letters**, v. 0, n. 0, p. 1–14, 2019.

SILVA, AM et al. Efeito da ingestão de Bifidobacterium longum na salmonelose experimental em camundongos. **Journal of Applied Microbiology** , v. 97, n. 1, p. 29-37, 2004.

SZCZESNIAK, A. S. Classification of textural characteristics. **J. Food Sci**. V. 28, n.4, p. 385-389, 1963.

TOMAR, O. The effects of probiotic cultures on the organic acid content, texture profile, and sensory attributes of Tulum cheese. **International Journal of Dairy Technology**, v. 72, n. 2, p. 218–228, 2019.

VINDEROLA, C.G, REINHEIMER, J.A Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. **Food Res Int**, 36(9-10):895-904, 2002, DOI: https://doi.org/10.1016/s0963-9969(03)00098-x.

VEISSEIRE P, BONNET M, SARAOUI T, POUPET C, CAMARÈS O, GACHINAT M, CALLON C, FEBVRE G, CHASSARD C, BORNES S. Investigation into In Vitro and *In Vivo Caenorhabditis elegans* Models to Select Cheese Yeasts as Probiotic Candidates for their Preventive Effects against *Salmonella* Typhimurium. **Microorganisms**. 2020 Jun 18;8(6):922. DOI: 10.3390/microorganisms8060922. PMID: 32570901; PMCID: PMC7356738.

YATES, M & DRAKE, M. A. Texture properties of Gouda cheese. **Journal of Sensory Studies**. Vol. 22. P. 493 – 506, 2007.

ZIVKOVIC, M. *et al.* Evaluation of the probiotic potential of yeasts isolated from traditional cheeses manufactured in Serbia and Croatia. **Journal of Intercultural Ethnopharmacology**, v. 4, n. 1, p. 12, 2014.

ARTIGO 2: Evaluation of the safety of *Kluyveromyces lactis* and *Torulaspora delbrueckii* and their survival to freeze-drying

ABSTRACT

The consumption of probiotic fermented foods has increased in recent years due to their health benefits and efficacy, contributing to the modification of the intestinal microbiota promoting immune stimulation. The production of these foods is carried out mainly with starter cultures for precise fermentation and in adequate quantities for them to be ingested. Recently, the use of yeasts in this context has been widely studied. *K. lactis* and *T. delbrueckii* are known due to their ability to produce volatile compounds in addition to being promising in the fermentation of dairy substrates and potential probiotic candidates. The objective of this work was to analyze the production of extracellular enzymes through proteolytic and lipolytic activity. Evaluate the safety of yeasts in terms of pathogenicity factors by gelatinase, DNase, and hemolysis tests. Check the viability of *K. lactis* B10 and *T. delbrueckii* B14 after being preserved by freeze-drying during 120 days of storage at room temperature and in the 4°C. Yeasts did not show proteolytic activity and only lipolytic activity. The safety assessment showed that the none isolates produced gelatinase, DNase, and hemolysis activity. The yeasts remained stable when conditioned at 4°C temperature. The plate counts showed that the viability of the 4°C temperature was 10^5 CFU / ml at the end of the process, while at room temperature at 60 days the population was 10^4 CFU / ml. Lyophilized yeasts when submitted to chemically simulated conditions of the gastrointestinal tract remained viable. These results demonstrate that the preservation method used was adequate while maintaining the chemical, physical and microbiological properties.

Keyword: Probiotic. Viability. Security. Freeze-drying.

1. INTRODUCTION

In recent years, the functional product's market has increased, among them, the use of probiotics that are defined as living microorganisms that beneficially affect the host, altering the native microbiota, promoting immune stimulation, that is, improving the host's reaction to intestinal diseases (GHANY, 2015). In the food industry, dairy products are the matrices most used as a vehicle for these microorganisms.

The use of probiotic yeasts is still limited and only *Saccharomyces boulardii* and *Saccharomyces cerevisiae* are commercialized (MARTINS et al., 2009). Recent research on the use of other yeast species or genera has been elucidated based on *in vivo* and *in vitro* tests.

In this context, these microorganisms have attractive properties such: as survival during passage through the human gastrointestinal tract, tolerance to low pH, resistance to bile salts, production of antimicrobial substances that mitigate the proliferation of pathogens in the intestinal lumen, being able to resist antibiotics, being ideal to be administered to patients who make continuous use of the drug, in addition to promoting immune modulation (SUVARNA et al., 2018).

Some strains have shown probiotic potential such as *Torulaspora delbrueckii* (ANDRADE et al., 2017, ANDRADE et al, 2019), *Debaryomyces hansenii* (FERREIRA, VILJOEN; 2003), *Yarrowia lipolytica* (CEUGNIEZ et al., 2017; FERREIRA, VILJOEN; 2003) and the genus *Kluyveromyces* (FADDA et al., 2017; ANDRADE et al., 2017, ANDRADE et al., 2019, OLIVEIRA et al., 2019).

For a microorganism to be considered probiotic, many requirements must be met, among them, the most important is survival in the gastrointestinal environment. It must be stable, safe, and viable in food products (SUVARNA et al., 2018). Yeasts are responsible for several metabolic reactions such as lipolytic and/or proteolytic activity that can be more pronounced in some products, producing compounds that are essential for quality, flavor, and aroma, being interesting to use as inoculum in the food industry (ZHANG et al., 2018; KARIMI; SOHRABVANDI; MORTAZAVIAN, 2012; COLLINS; MCSWEENEY; WILKINSON, 2003; LARSEN; JENSEN, 1999).

The probiotic strains modify the biochemical profile improving the organoleptic characteristics of the products, due to the different proteolytic and lipolytic patterns as reported in the work by Patrignani et al., (2019). Probiotic formulations must guarantee the maintenance of viability and microbial function, emphasizing the importance of their concentrations to confer benefits to the host (SUVARNA et al., 2018).

Thus, freeze-drying has been an effective method of cell preservation and survival during storage for long periods, microbial integrity is essential to obtain beneficial effects throughout the intestinal tract. After the product is frozen at temperatures below -80 °C, it is subjected to negative pressure (vacuum), causing all the water in the product to be removed by sublimation, preserving the physical-chemical and microbiological characteristics of the sample (BOLLA et al., 2011).

Due to the adverse conditions during the process, cryoprotectants are used, such as carbohydrates and protein substances, amongst them milk, ensuring the survival of microorganisms during freeze-drying and storage. Thus, the preservation of probiotic strains and the maintenance of their properties are essential in the industrial context for their use as a probiotic supplement or as starter cultures (MARTINS et al., 2009).

The objective of the present study was to evaluate the safety aspects, the production of extracellular enzymes, and the freeze drying of the yeasts *K. lactis* B10 and *T. delbrueckii* B14 that can be used as a probiotic supplement or starter culture in the food industry.

2. MATERIALS AND METHODS

2.1 PREPARAÇÃO DOS MICRORGANISMOS

The yeasts in the present study were previously isolated from the production of Canastra cheese obtained by Andrade et al. (2017), being stored in glycerol at -20 ° C in the Microbiology laboratory of the Federal University of Lavras.

K. lactis B10 and *T. delbrueckii* B14 were reactivated in 1 mL of YPD (2% peptone, 2% glucose, and 1% yeast extract) incubated at 28 ° C for 24 hours, triggering cell growth in increasing volumes of medium to obtain the desired number of cells. The biomass obtained was used for the tests.

2.2 PRODUCTION OF EXTRACELLULAR ENZYMES

2.2.1 Lipolytic activity

Lipolytic activity was evaluated using as a substrate the tributyrin-based agar medium enriched with 2% tributyrin. *K. lactis* B10 and *T. delbrueckii* B14 were reactivated in YPD medium at 28 ° C for 24 hours, then plated on tributyrin agar medium incubated at 28 ° C for 72h. As a positive control, the bacterium *Pseudomonas fluorescens* was used, reactivated in the BHI medium at 25 ° C for 24h, and then plated on tributyrin-based agar incubated at 25 ° C for 3 days (MUNSCH-ALATOSSAVA & ALATOSSAVA 2006; BRAUN et al 2001). The reaction was characterized as positive by forming translucent halos around the colony indicating that the microorganism has the enzyme lipase. Lipases catalyze the hydrolysis reaction of triacylglycerol's converting to free fatty acids and glycerol.

2.2.2 Proteolytic activity

Proteolytic activity was determined according to the methodology described by Munsch-Alatossava & Alatossava (2006) in the milk agar medium, supplemented with 5% skimmed milk powder. *K. lactis* B10 and T. *delbrueckii* B14 were reactivated in YPD medium at 28 ° C for 24h. As a positive control, *Pseudomonas fluorescens* was used, reactivated in the BHI medium at 25 °C for 24h, afterward the microorganisms were plated on milk agar medium, incubated at their optimal growth temperature maintained for 72 h. The reaction was characterized as positive with the formation of clear halos around the colonies.

2.3 SECURITY ASSESSMENT

2.3.1 Gelatinase activity

The ability of a microorganism to produce it is observed by biochemical tests. Thus, *K. lactis* B10 and *T. delbrueckii* B14 were reactivated in YPD. As a positive control, the strain *Staphylococcus aureus* 3165 was used reactivated in the BHI medium, maintained at 24 hours. The gelatin turbidity and liquefaction test were performed as described by Faddin, (2000) using the following concentrations in g / L, 3 g of meat extract; 5.0 g of peptone; 120.0 g of gelatin. After reactivation, 5 µl of inoculum was added to the test tubes containing the gelatin medium.

The test tubes were kept at 25 °C and 35 °C for 15 days. Besides, the yeasts were also kept at their optimum temperature at 28 °C and 37 °C, simulating body conditions. Every 24 hours the samples were analyzed for turbidity. The liquefaction was observed through the ice bath maintained for 2 hours, which allows the gelatin to solidify, if the microorganism has the enzyme gelatinase, there is the liquefaction of the medium, characterizing the reaction as positive.

2.3.2 DNase test

The DNase production test assesses whether the microorganism has the deoxyribonuclease enzyme, which is responsible for the degradation of nucleic acid (DNA). The production of this enzyme was analyzed by streak cultivation after 24 hours of reactivation in the Log phase of the growth of microorganisms on the DNase agar medium (HiMedia). In this way, *K. lactis* B10 and *T. delbrueckii* B14 were incubated at a temperature of 28 ° C (optimal temperature of the microorganism) and 37 ° C (body temperature) for 72 hours. As a positive control, the strain *Staphylococcus aureus* Saur ATCC 33592 was used, maintained at 37 ° C for 72 hours. After incubation, 0.1% hydrochloric acid solution was added to the plates for 5 minutes. The reaction was characterized as positive by forming clear halos around the colonies (SYAL; VOHRA, 2013; GUPTA; MALIK, 2007).

2.3.3 Hemolysis Test

The yeast strains were previously plated in YPD medium, incubated at 28 ° C, for 48 hours to obtain pure and isolated colonies. Subsequently, the colonies were transferred to Blood Agar Base plates (HiMedia) supplemented with 5% fresh bovine blood. As a positive control, the bacteria ATCC *Streptococcus pneumoniae* 40619 was used. The plates were incubated for 3 days at 37 °C and 28 °C (FADDA et al., 2017).

2.4 PRESERVATION OF *K. LACTIS* E *T. DELBRUECKII* BY FREEZE-DRYING

2.4.1 Preparation of products for freeze-drying procedure

K. lactis B10 and *T. delbrueckii* B14 were cultured individually until reaching the desired amount of cells for freeze drying following the adapted methodology described by Pietrowski et al., (2012) and Bolla et al., (2011). Thus, 20 µL of each yeast stored at -20 ° C was reactivated in 1 ml YPD, incubated at 28 ° C for 24 h. This culture was transferred to an Erlenmeyer flask containing 10 ml of the same medium and incubated under the same conditions. Subsequently, they were transferred to 100 ml to obtain a population of approximately 10^7 CFU / ml. Then, the cultures were centrifuged at 9000 rpm for 5 min and washed twice with sterile distilled water. After centrifugation and washing, the yeast biomass was resuspended in 500 µL of sterile UHT milk or UHT milk. The samples were frozen at -80 ° C for approximately 8h in ampoules and successively taken to the lyophilizer with an initial temperature of −56 ° C and an initial vacuum of 1335 Vac and 66 µHg. Lyophilized cells were stored at 4 ° C and room temperature for 120 days. After freeze drying and at different time intervals (0, 15, 30, 60, 90, 120) the samples were rehydrated to the original volume with sterile deionized water and plated for counting viable cells.

2.4.2 Assessment of the viability of strains

The viability of the strains was determined by the number of colony-forming units per mL (CFU / mL). The samples were subjected to serial dilution (10^{-1} to 10^{-5}) prepared in 0.1% tryptone and plated in YPD, incubated at 28 ° C for 48 hours (BOLLA et al., 2011).

2.5 SIMULATION OF GASTROINTESTINAL CONDITIONS

The yeasts in the present study were previously reactivated in 1 mL of YPD (1% yeast extract; 2% peptone; 2% glucose) at 28 ° C for 24 h. Cell growth was triggered by increasing supplementation of YPD medium, until the desired amount of 10^8 CFU / mL microbial biomass was obtained for freeze drying and consequently the simulation of the gastrointestinal tract, to assess their viability as a commercial probiotic product (Andrade et al., 2017).

After freeze drying, the microbial cells were subjected to simulated conditions of the gastrointestinal tract, containing the synthetic gastric and duodenal juice. The lyophilized of each yeast was initially inoculated in 10 ml of 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) incubated at 37 ° C with shaking. After 90 min, 17.5 mL of synthetic duodenal juice (6.4 g / L $NaHCO_3$, 0.239 g / L KCl, 1.28 g / L NaCl, 0.1% pancreatin, 10% ox-bile, adjusted pH to 7.4 with 5M HCl) were added and again stirred at 37 ° C, 150 rpm for 180 min simulating the passage through the intestinal tract. The

survival rate of the strains was evaluated at 0 (T0), 90 (T1) and 270 (T2) min, using the surface plating technique in the YPD medium, incubated at 37 ° C for 48 h (Fadda et al 2017).

3. RESULTS AND DISCUSSION

3.1 PRODUCTION OF EXTRACELLULAR ENZYMES

3.1.1 Lipolytic activity

The reaction was positive with the formation of light halos around the colonies, showing that both the yeast *K. lactis* B10 and *T. delbrueckii* B14 have lipolytic activity with the enzyme lipase. Lipases catalyze the hydrolysis reaction of triacylglycerols converting to free fatty acids and glycerol, being responsible for the aggregation of the flavor and aroma of many dairy products (W PARK; JEANJULIEN; SIDDIQUE, 2017; DESMASURES et al., 2015; MCSWEENEY et al., 2000). According to Karimi et al., (2012), the lipolytic activity of probiotic strains acts in improving the taste of dairy products resulting in a greater sensory appreciation.

Lipolytic activity is associated with the production of flavoring compounds, among them, in the cheese maturation process (DESMASURES et al., 2015; EL SODA, 1993; OLSON, 1990). In cheeses, these acids contribute to characterize them in different types, generating aromas that differentiate the different types of cheese (DESMASURES et al., 2015; SIMILI, LIMA, 2007).

3.1.2 Proteolytic activity

The yeasts *K. lactis* B10 and *T. delbrueckii* B14 did not show proteolytic activity for casein when compared to the positive control. According to Chamba et al., (2004), the proteolytic activities can be variable according to the yeast lineage, being caseinolytic, aminopeptity, or carboxypeptity. Thus, the secretion of proteases is dependent on factors, including the composition of the medium. This enzyme has different degrees of specificity in addition to having specific functionality as observed in the works of Ray et al., (1992).

Fadda et al., (2004) observed in their studies that among the strains sampled *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Candida zeylanoides*, and *Geotrichum candidum* exhibited this activity on casein agar although other strains showed only lipolytic activity, in agreement with the present study.

3.2 SECURITY ASSESSMENT

3.2.1 Gelatinase activity

The gelatinase activity test was performed for 15 days, in which the yeasts were incubated and a positive control at temperatures of 25 °C and 35 °C, as described by Faddin (2000) and at temperatures of 28 °C and 37 °C. The test was observed every 24 hours. The gelatinase enzyme is produced mainly by pathogenic microorganisms being able to degrade the components of the extracellular matrix, as well as macromolecules, including casein, gelatines of type I, II, IV, and V, fibronectin, and proteoglycan (ZHAO et al., 2011).

K. lactis B10 and *T. delbrueckii* B14 did not show gelatinase activity, although they have grown in all the mentioned temperature ranges. According to Syal et al., (2013), the absence of this enzyme is a safety indicator to be used as probiotics in food. Only the microorganism used as a positive control, *Staphylococcus aureus* 3165, showed turbidity of the medium and the liquefaction capacity.

3.2.2 DNase test

K. lactis B10, *T. delbrueckii* B14, and *Staphylococcus aureus Saur* ATCC 33592 were plated on DNase agar incubated at 28 ° C and 37 ° C for 72h. Yeasts grew only at 28 ° C.

To read the plates, 1 mL of 1N hydrochloric acid was added. Only the positive control containing *Staphylococcus aureus Saur* ATCC 33592 showed the formation of clear halos around the colonies. Thus, yeasts do not show deoxyribonuclease activity at any of the tested temperatures.

According to Hasegawa et al., (2010), the production of extracellular DNase is closely associated with pathogenicity factors since they allow the dissemination of the pathogen in addition to contributing to the evasion of the immune response. Among the yeast genera that exhibit this activity, Cryptococcus sp., Endomycopsis sp., Tremella, and Rhodotorula sp. (SÁNCHEZ; COLOM, 2010). According to Syal et al., (2013), the absence of this production is an indication of safety to be used as probiotics.

3.2.3 Hemolysis test

Hemolytic activity is characterized by premature destruction of red blood cells caused by the rupture of the plasma membrane, resulting in the release of hemoglobin, one of its causes is due to infections by microorganisms.

The hemolytic reaction is classified into α-hemolysis, β-hemolysis, and γ-hemolysis, according to the intensity of the breakdown of red blood cells. The α -hemolysis is characterized by the partial loss of red blood cells forming green halos around the colonies. The total loss of

red blood cells is classified as β-hemolysis, forming clear hydrolysis halos around colonies. The absence of halos around the colonies is classified as γ-hemolysis, without the enzyme.

For the hemolysis test, the yeasts in the present study were incubated at 28 ° C and 37 ° C together with the positive control. After 72 hours of yeast incubation at 28 °C, they formed well-defined streaks. The positive control was kept under incubation at 37 ° C for 72 hours, making the formation of halos around the colonies more evident.

Yeasts were also incubated at 37 ° C and maintained for 72 hours, so they did not show enzymatic activity (hemolysin) following the findings of Fadda et al (2017). No late hemolysin activity is known after 72 hours. Therefore, the use of these yeasts as probiotic cultures does not pose a risk for hemolytic activity.

3.3 PRESERVATION OF *K. LACTIS* E *T. DELBRUECKII* BY FREEZE-DRYING

The lyophilized cells of the yeasts *K. lactis* B10 and *T. delbrueckii* B14 obtained were analyzed for survival rate during storage for 120 days at 4°C and room temperatures.

After freeze-drying and at different intervals of days (0, 15, 30, 60, 90, and 120) the samples were rehydrated to the original volume with sterile deionized water and plated for counting viable cells.

The viability of the *K. lactis* B10 and *T. delbrueckii* B14 cells after freeze-drying presented successively counts of 3.04 x 10^7 CFU / ml and 3.44 x 10^7 CFU / ml. The initial populations obtained before the process were 1.36 x 10^7 CFU / ml and 1.96 x 10^7 CFU / ml. Thus, the yeasts in the present study were able to withstand the stress submitted during the freezing process at -80°C, subsequently freeze-drying in which the material dehydrates due to sublimation at low temperatures under vacuum.

According to Bolla et al., (2011) cultures with concentrations equal to or greater than 10^7 CFU / ml for freeze-drying, have a greater chance of success. These concentrations ensure that sufficient cells are remaining after the process and, consequently, long-term storage, providing successful reactivation of the strain.

Yeasts stored at room temperature showed a significant reduction in survival rates after 60 days, with populations of approximately 10^4 CFU / ml. In contrast, lyophilized yeasts kept at 4°C temperature, the results of yeast plate counts show population stability (10^6 CFU / ml) as reported by Pietrowski et al., (2012). Although the maintenance of the samples at room temperature was short-term, they confer certain advantages in storage as mentioned by Pietrowski et al., (2012) for eliminating the cost of electricity for the maintenance of the product, as well as possible risks of lack of energy and consequent loss of material.

The *K. lactis* B10 and *T. delbrueckii* B14 count preserved by freeze-drying at 4 ° C showed values in the range of 10^5 and 10^6 CFU / ml (Table 1). It is worth mentioning that the population was not significantly different from the 60 to 120 days of storage. However, significant differences were found in the samples kept at room temperature after 2 months.

This study shows the development of a potential lyophilized probiotic supplement that can be preserved and stored for a longer period. The protective effect of milk contributed so that cell viability was not compromised, in line with the work of Bolla et al., (2011). The permanence of the viability of the strains and functionally active during long-term conditioning is a strong requirement for potential probiotics.

Table 1: Viability expressed on a logarithmic scale and standard deviation of *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 in 120 days of storage at 4°C and room temperatures.

	T0		T15				T30				T60				T90				T120			
	Room temperature		4°C		Room temperature		4°C		Room temperature		4°C		Room temperature		4°C		Room temperature		4°C		Room temperature	
B10	7.48	±0.37	7.33	±0.22	6.40	±0.09	7.27	±0.27	6.28	±0.44	7.00	±0.21	3.81	0.00	6.67	±0.33	2.62	0.00	6.41	±0.29	2.09	0.00
B14	7.54	±0.34	7.73	±0.30	6.33	±0.10	7.12	±0.19	5.59	±0.08	6.53	±0.21	3.68	±1.66	6.90	±0.49	2.59	±0.42	5.86	±0.21	2.53	0.00

3.4 LIOPHILIZATION PROCESS AND SIMULATION OF GASTROINTESTINAL CONDITIONS

Yeasts remained viable throughout the process of freeze-drying and simulation of gastrointestinal conditions. The initial populations of *K. lactis* B10 cells before the freeze-drying process had counts of 2.23 X 10^8 CFU / ml. After the freeze-drying process, a population of 8.89 x 10^7 CFU / ml was found (Figure 1). When gastrointestinal conditions were simulated and added to gastric juice, at 90 minutes the survival rate obtained was 97.98% (Figure 2). After being subjected to passage through the duodenal juice, the final population after 180 min was 96.60% (Figure 2).

The populations of *T. delbrueckii* B14 cells remained stable throughout the process. The count obtained initially was 2 x 10^8 CFU / ml. After freeze-drying, its viability was 3 x 10^8 CFU / ml (Figure 1). At 90 min, after the addition of gastric juice, the survival rate found was 100% (Figure 2 and Table 2). While the rate obtained when exposed to duodenal juice was 97.75% about the initial population (Figure 2 and Table 2).

One of the requirements of a probiotic product is its ability to withstand the adversities found in the host organism, such as crossing the gastrointestinal tract. Among them, the first barrier to be faced is the gastric juice present in the stomach, which has a low pH ranging from 1.5 to 3.5, acting as a defense mechanism of the body against various pathogens (HELMY et al., 2019; OLIVEIRA et al., 2019). Thus, the probiotic strain must remain viable during the crossing of stomach acid and the action of lytic enzymes present there.

Then, the next barrier to be faced is resistance to pancreatic secretion, which contains bicarbonate ions and digestive enzymes making up the duodenal intestine. To simulate these conditions, as well as the enzymatic actions occurring in the duodenal juice, commercial pancreatin (Creon®) was added. Subsequently, these strains were exposed to bile, as they resist bile salts, they can adhere to the intestinal epithelium (ANDRADE et al., 2019; OLIVEIRA et al., 2019; HELMY et al., 2019).

Thus, the survival rate of *K. lactis* B10 after passing through the gastrointestinal tract showed a decrease of 3.4% about its initial population, in agreement with the works present in the literature, which report declines of the population of 18.5% (Andrade et al., 2019), 24.67% (Oliveira et al., 2019), 55% (Ceugniez, 2017), 87.4% (Fadda et al., 2017). While the survival of *T. delbrueckii* B14 declined only 2.25% after the simulation of gastrointestinal conditions, as also evidenced by Andrade et.al.,(2019) declines of 15.33%.

The survival of these yeasts at a low pH present in the simulated conditions can be justified due to their adaptation to the Canastra cheese from which they were isolated combined with a naturally acidic substrate due to the coexistence of lactic bacteria as seen in the works by Andrade et al., (2017) and Andrade et al., (2019).

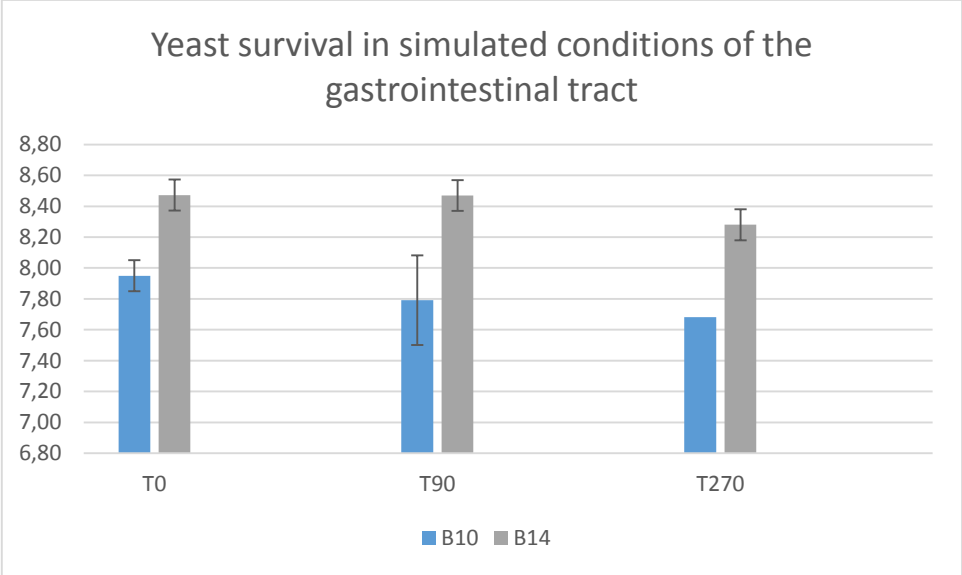
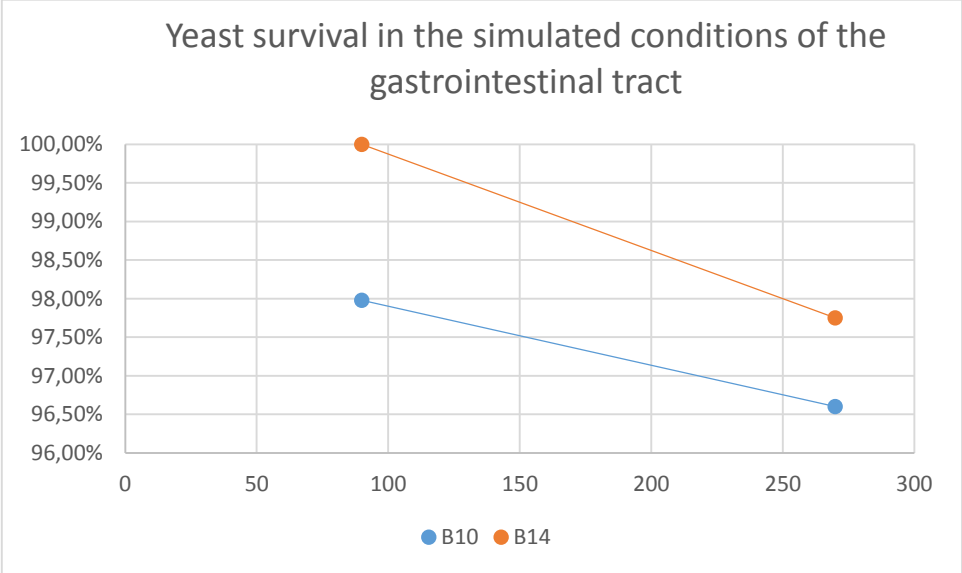


Figure 1 - Survival of the yeasts *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 in log / mL (mean ± experimental standard deviation) over time in the simulated conditions of the gastrointestinal tract.



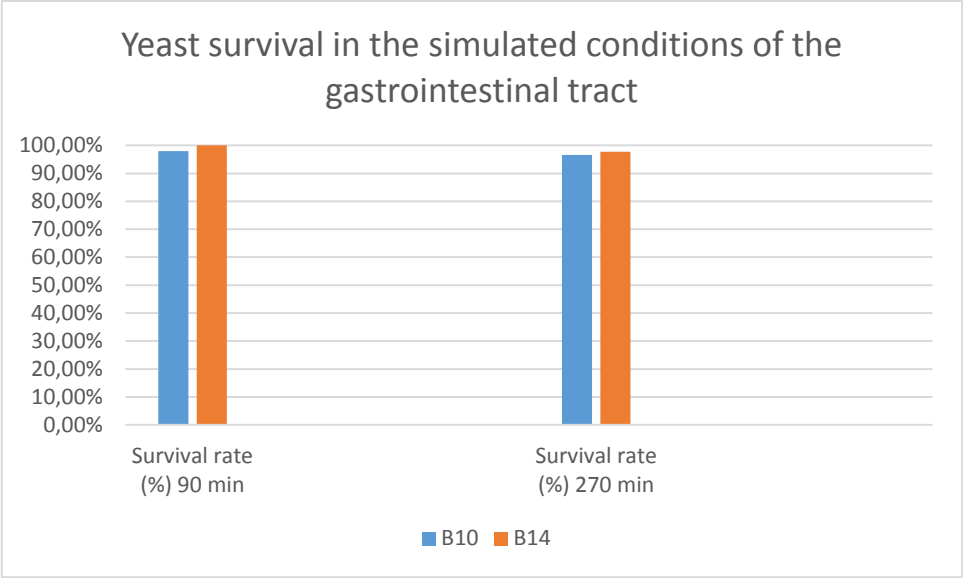


Figure 2 - The survival rate of the yeasts *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 by time in the simulated conditions of the gastrointestinal tract.

Table 2: The survival rate of the yeasts *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 by time in the simulated conditions of the gastrointestinal tract.

Yeasts	**Survival rate (%) 90 min**		**Survival rate (%) 270 min**	
B10	97.98%	±0.32	96.60%	±0.29
B14	100%	±0.34	97.75%	±0.12

CONCLUSION

Yeasts exhibited advantageous characteristics to be used as probiotics, that is, safe to be ingested due to the absence of gelatinase, DNase, and hemolysis activity. Thus, they are promising candidates for their use as starter cultures in the fermentation of dairy substrates due to the presence of lipolytic activity that contributes to the sensory characteristics of the final product. *K. lactis* and *T. delbrueckii* showed interesting properties due to the preservation capacity by the freeze-drying method and later the passage through the gastrointestinal tract. Based on these conditions, this study suggests its possible use in the food industry for the development of functional foods.

REFERÊNCIAS

ANDRADE, R. P. *et al*. Yeasts from Canastra cheese production process: Isolation and evaluation of their potential for cheese whey fermentation. **Food Research International**, v. 91, p. 72–79, 2017.

ANDRADE, R. P. *et al*. Survival of Kluyveromyces lactis and Torulaspora delbrueckii to simulated gastrointestinal conditions and their use as single and mixed inoculum for cheese production. **Food Research International**, v. 125, n. March, p. 108620, 2019.

BOLLA, P. A. et al. Effect of freeze-drying on viability and *in vitro* probiotic properties of a mixture of lactic acid bacteria and yeasts isolated from kefir. **Journal of Dairy Research**, v. 78, n. 1, p. 15–22, 2011.

BRAUN, P., BALZER, G., & FEHLHABER, K. Activity of bacterial lipases at chilling temperatures. **Food Microbiology**, 18(2), 211–215, 2001.

CHAMBA, J.-F., IRLINGER, F., Secondary and Adjunct Cultures, in **Cheese: Chemistry, Physics, and Microbiology**, P. F. Fox, P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee, Eds., Oxford, UK, **Elsevier**, pp. 191-206, 2004.

CEUGNIEZ, A. et al. Interactions between Kluyveromyces marxianus from cheese origin and the intestinal symbiont Bacteroides thetaiotaomicron: Impressive antioxidative effects. **Lwt**, v. 81, p. 281–290, 2017.

COLLINS, Y. F.; MCSWEENEY, P. L. H.; WILKINSON, M. G. Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. **International Dairy Journal**, v. 13, n. 11, p. 841–866, 2003.

DESMASURES, N et al. Smear Ripened Cheeses - **Diversity, dynamics, and functional role of actinomycetes on European smear-ripened cheeses**. v.1, p. 1-18, 2015.

EL SODA, M. A. The role of lactic acid bacteria in accelerated cheese ripening. **FEMS Microbiology**. Rev12, p. 239–252, 1993.

FADDA, M. E. et al. Occurrence, and characterization of yeasts isolated from artisanal Fiore Sardo cheese. **International Journal of Food Microbiology**, v. 95, n. 1, p. 51–59, 2004.

FADDA, M. E. et al. *In vitro* screening of Kluyveromyces strains isolated from Fiore Sardo cheese for potential use as probiotics. **LWT - Food Science and Technology**, v. 75, p. 100–106, 2017.

FADDIN, J.F.M. Biochemical Tests for Identification of Medical Bacteria, V. 3, p- 129-131, **Philadelphia: Lippincott Williams & Wilkins**, 2000.

FERREIRA, A. D., & VILJOEN, B. C. Yeasts as adjunct starters in matured Cheddar cheese. **International Journal of Food Microbiology**, 86(1–2), 131–140, 2003

GHANY, T. M. A. Safe Food Additives : A Review. **International Journal of Life Science and Chemistry**, v. 32, n. 1, p. 402–437, 2015.

GUPTA, H.; MALIK, R. K. Incidence of virulence in bacteriocin-producing enterococcal isolates. **Le Lait**, v. 87, n. 6, p. 587–601, 2007.

HASEGAWA, T. et al. Characterization of a virulence-associated and cell-wall-located DNase of Streptococcus pyogenes. **Microbiology**, v. 156, n. 1, p. 184–190, 2010.

HELMY, E. A. et al. Evaluation of potentially probiotic attributes of certain dairy yeast isolated from buffalo sweetened Karish cheese. **Heliyon**, v. 5, n. 5, p. e01649, 2019.

KARIMI, R.; SOHRABVANDI, S.; MORTAZAVIAN, A. M. Review Article: Sensory Characteristics of Probiotic Cheese. **Comprehensive Reviews in Food Science and Food Safety**, v. 11, n. 5, p. 437–452, 2012.

LARSEN, M. D.; JENSEN, K. The effects of environmental conditions on the lipolytic activity of strains of Penicillium roqueforti. **International Journal of Food Microbiology**, v. 46, n. 2, p. 159–166, 1999.

MARTINS, F. S. et al. Effects of yeast probiotic formulation on viability, revival, and protection against infection with Salmonella enterica ssp. enterica serovar Typhimurium in mice. **Letters in Applied Microbiology**, v. 49, n. 6, p. 738–744, 2009.

MCSWEENEY, P. L. H., SOUSA, M. J. Biochemical pathways for the production of flavor compounds in cheeses during ripening: A review. **Lait** 80, p. 293–324, 2000.

MUNSCH-ALATOSSAVA, P.; ALATOSSAVA, T. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. **Microbiological Research**, v. 161, n. 4, p. 334-346, 2006.

OLIVEIRA, D. R. et al. Selection of potentially probiotic Kluyveromyces lactis for the fermentation of cheese whey-based beverage. **Annals of Microbiology**, v. 69, n. 13, p. 1361–1372, 2019.

OLSON, N. F. The impact of lactic acid bacteria on cheese flavor. **FEMS Microbiology**. Rev 87, p. 131–148, 1990.

PATRIGNANI, F. et al. Use of Lactobacillus crispatus to produce a probiotic cheese as potential gender food for preventing gynaecological infections. **PLoS ONE**, v. 14, n. 1, p. 1–19, 2019.

PIETROWSKI, G.D., SANTOS, C.M., SAUER, E., WOSIACKI, G., & NOGUEIRA, A. (2012). Influence of fermentation with Hanseniaspora sp. yeast on the volatile profile of fermented apple. **Journal of agricultural and food chemistry**, *60 39*, 9815-21.

PITINO, I. et al. Survival of Lactobacillus rhamnosus strains inoculated in cheese matrix during simulated human digestion. **Food Microbiology**, v. 31, n. 1, p. 57–63, 2012.

RAY, M. K. et al. Extracellular protease from the antarctic yeast Candida humicola. **Applied and Environmental Microbiology**, v. 58, n. 6, p. 1918–1923, 1992.

SÁNCHEZ, M.; COLOM, F. Actividad DNasa extracelular en Cryptococcus neoformans y

Cryptococcus gattii. **Revista Iberoamericana de Micologia**, v. 27, n. 1, p. 10–13, 2010.

SIMILI, F. F; LIMA, M. L. P; Como os alimentos podem afetar a composição do leite das vacas. **Pesquisa & Tecnologia**, vol. 4, n.1 Jan-Jun 2007; ISSN 2316-5146. Disponível em: <http://www.aptaregional.sp.gov.br/> Acesso: 30 de Abril de 2019

SUVARNA, S. et al. Potential probiotic characterization, and effect of encapsulation of probiotic yeast strains on survival in simulated gastrointestinal tract condition. **Food Science and Biotechnology**, v. 27, n. 3, p. 745–753, 2018.

SYAL, P.; VOHRA, A. PROBIOTIC POTENTIAL OF YEASTS ISOLATED FROM TRADITIONAL INDIAN FERMENTED FOODS. **International Journal of Microbiology** v. 5, n. 2, p. 390–398, 2013

W PARK, Y.; JEAN JULLIEN, C.; SIDDIQUE, A. Factors Affecting Sensory Quality of Goat Milk Cheeses: A Review. **Advances in Dairy Research**, v. 05, n. 03, 2017.

ZHANG, Z., Lv, J., Pan, L. *et al.* Roles and applications of probiotic *Lactobacillus* strains. ***Appl Microbiol Biotechnol* 102,** 8135–8143, 2018.

ZHAO, Q. et al. Ds-echinoside A, a new triterpene glycoside derived from sea cucumber, exhibits antimetastatic activity via the inhibition of NF-κB-dependent MMP-9 and VEGF expressions. **Journal of Zhejiang University: Science B**, v. 12, n. 7, p. 534–544, 2011.