UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO TECNOLÓGICO
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE ALIMENTOS

Adriana Dantas

Leite sem lactose como agente encapsulante de probiótico pelo método de *spray drying* e seu comportamento em processos de crioconcentração

Florianópolis - SC

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Tese submetida ao Programa de Pós-Graduação em Engenharia de Alimentos da Universidade Federal de Santa Catarina, como requisito à obtenção do título de Doutora em Engenharia de Alimentos.
Orientadora: Profª. Drª. Elane Schwinden Prudêncio
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Florianópolis - SC
2021

Ficha de identificação da obra elaborada pelo autor,
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DANTAS, ADRIANA
Leite sem lactose como agente encapsulante de probiótico pelo método de spray drying e seu comportamento em processos de crioconcentração / ADRIANA DANTAS ; orientador, Elane Schwinden Prudêncio, coorientador, Eduard Hernandez Yañez, 2021.
248 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro Tecnológico, Programa de Pós-Graduação em Engenharia de Alimentos, Florianópolis, 2021.

Inclui referências.

1. Engenharia de Alimentos. 2. leite sem lactose. 3. encapsulação de probiótico. 4. crioconcentração. 5. simulação gastrointestinal in vitro. I. Schwinden Prudêncio, Elane . II. Hernandez Yañez, Eduard. III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Engenharia de Alimentos. IV. Título.

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em Engenharia de Alimentos pelo Programa de Pós-graduação em Engenharia de Alimentos.

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Florianópolis, 2021.

Dedico aos meus pais, Maria e Jorge, que me mostraram uma versão belíssima do amor sem medidas, carinho, paciência, amizade, e dedicação. Mal consigo expressar a felicidade de ter compartilhado com eles minha vida, meus sonhos, as incertezas, e as experiências.

AGRADECIMENTOS

À Universidade Federal de Santa Catarina e ao Programa de Pós-Graduação em Engenharia de Alimentos, pela oportunidade de realizar o doutorado.

À minha querida orientadora Profa. Dra. Elane Schwinden Prudêncio. Obrigada pelo apoio, positividade e dedicação ao longo desses anos. Por todas as conversas, os deliciosos cafés, e os sábios conselhos.

À Universitat Politècnica de Catalunya (ES), pela oportunidade de usufruir de suas instalações. Aos funcionários, técnicos e professores dessa instituição, pela ajuda e receptividade.

Ao Prof. Dr. Eduard Hernández, por aceitar a proposta de intercâmbio e de cotutela. Sou grata pela sua paciência em ensinar, e pela preocupação e cuidado. Obrigada por sempre me inserir no mundo acadêmico, me apresentando à grandes pesquisadores. Nesse contexto, agradeço à Profa. Dra. Mónica Blanco, por seu tempo despendido na área de estatística, ao Prof. Dr. Patricio Orellana Palma pela contribuição nos ensaios de centrifugação, e à Dra. Maria Isabel Mora Garrido, por ser solícita durante minha pequena estadia na cidade de Vic.

Aos meus colegas de pesquisa do Laboratório de Leite e Derivados, aos presentes e aos que já partiram, agradeço pela colaboração técnica, pelas risadas e companheirismo. Em especial à Silvani Verruck pela grande ajuda nas análises, por ser solícita e compartilhar seu conhecimento pacientemente; e à Maria Helena, que me auxiliou na área de crioconcentração e se tornou uma estimada amiga.

Ao professor Prof. Dr. Carlos Eduardo Maduro de Campos, pelos ensinamentos na área de difração de raios X e espectroscopia raman.

À Central de Análises do Departamento de Engenharia Química e Engenharia de Alimentos da UFSC, pelo auxílio nas análises de propriedades texturais.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) pelo apoio financeiro (Código de Financiamento 001).

À empresa Christian Hansen, pela doação da cultura de *Bifidobacterium* BB-12.

À banca, por aceitar o convite de participação e contribuição.

Aos professores, funcionários e colegas do Departamento de Ciência e Tecnologia de Alimentos e do Departamento de Engenharia Química e Engenharia de Alimentos.

Aos meus pais Maria e Jorge, pelo apoio de sempre, por entenderem minha ausência, e pelos sábios ensinamentos. Eu me espelho em vocês e tenho um orgulho imensurável de tê-los como pais. Obrigada pelo amor, carinho, e cuidado.

Aos demais membros da minha família (irmãos, cunhados e sobrinhos), pelo constante incentivo e carinho. Do mesmo modo, agradeço a Deus por me conceder tamanha dádiva de tê-los como família.

Ao meu amado esposo Lindiomar Borges, que sempre esteve disposto a enriquecer este trabalho através dos seus conhecimentos de caracterização física de materiais. Obrigada pela paciência, amor, atenção, e por estar ao meu lado em todos os momentos.

Aos amigos do PPGEAL, em especial Aline Elias, Talyta Torres e Jéssica Fonseca. Sou grata pelo companheirismo, confiança, e pela troca de experiências durante o intercâmbio.

Aos meus queridos amigos que estão distantes; Samara Kelly, Cipriano, Juliane Gomes, Bianca Gomes, Letícia Volcov, Palloma Rodrigues, Ana Carolina Gomes, Adiléia Silva, Pedro Ferreira e Nataly Sousa. Agradeço pela torcida e preocupação. O passar do tempo não mudou o carinho e admiração que tenho por cada um de vocês.

À Deus, pela vida e por tantas oportunidades!

RESUMO

Estima-se que o quadro de intolerância à lactose (seja ela temporária ou permanente) aflige parte considerável da população mundial. Os números relatados na literatura variam entre 65 e 75%. Buscando criar alternativas para essa demanda, assim como simplesmente oferecer novas possibilidades para quem não deseja consumir lactose, este trabalho investigou o potencial do leite sem lactose em tecnologias emergentes e inovadoras. Nesse sentido, o trabalho experimental foi desenvolvido em três grandes etapas: Primeiramente, leite desnatado sem lactose foi utilizado como matriz de encapsulação de uma bactéria probiótica (*Bifidobacterium* BB-12). Não somente o leite, como também carboidratos prebióticos (oligofrutose e inulina) foram empregados no processo de microencapsulação por *spray drying*. Os pós obtidos foram sujeitos às análises de rendimento de encapsulação, viabilidade microbiana durante armazenamento (temperatura de refrigeração e temperatura ambiente), e sobrevivência do probiótico durante passagem pelo sistema gastrointestinal. Num segundo momento, os pós também foram caracterizados quanto às suas propriedades físico-químicas. Para isso, extensas avaliações foram feitas, dentre elas: estudo da cor; determinação da umidade, atividade de água e densidade; investigação das propriedades de sorção de água; análises térmicas; e morfologia dos pós. O maior rendimento de encapsulação (88,01%) e a maior viabilidade de bifidobactérias durante 120 dias de armazenamento a 25 °C foram observados para o pó oriundo de leite sem lactose com inulina. Por outro lado, o pó constituído de leite sem lactose e oligofrutose foi o que apresentou melhor sobrevivência do probiótico após passar por condições gastrointestinais simuladas *in vitro*. Do mesmo modo, ao avaliar todas as propriedades físico-químicas dos pós, foi observado que as formulações que continham os prebióticos foram as que resultaram em características mais desejáveis de estabilidade térmica, umidade e atividade de água. Contudo, não concluiu-se que o pó constituído unicamente de leite sem lactose tenha apresentado propriedades insatisfatórias e/ou inaceitáveis. Por exemplo, sob temperaturas de refrigeração, estabilidade microbiana também foi alcançada por 120 dias de estocagem. Igualmente, ao avaliar as propriedades térmicas, constatou-se que este pó foi estável até ~130 ºC. Em relação à sorção de água, suas características foram semelhantes às do pó obtido de leite com inulina; ou seja, condições de umidade relativa acima de 33% não são indicadas para manter a estabilidade físico-química desses pós ao longo do armazenamento. Foi por causa desse conjunto de informações que o pó probiótico à base de leite sem lactose foi escolhido (assim como os demais) para ser estudado na segunda parte desta tese: aplicação dessas micropartículas em um produto lácteo sem lactose. Este produto foi desenvolvido de forma a obter um leite fermentado tipo *skyr*, que é um alimento típico da Islândia. Se trata de um leite fermentado concentrado e que portanto, apresenta alto teor de proteína e quantidade desprezível de gordura. Assim, foram preparadas quatro formulações de leite fermentado concentrado: três delas contendo cada pó probiótico, e uma formulação contendo a bifidobacteria no estado livre (denominada amostra controle). Como já esperado, a inserção dos pós no leite fermentado modificou algumas propriedades, como o pH, acidez, e firmeza. Por causa dessas características, os produtos adicionados dos pós probióticos foram relatados como as melhores formulações para o desenvolvimento de um novo iogurte probiótico concentrado sem lactose. A terceira e última parte trabalhada nesta tese foi o emprego da crioconcentração no leite desnatado sem lactose. Foi uma etapa realizada em parceria com a Universitat Politècnica de Catalunya (Espanha). Investigou-se a crioconcentração do leite utilizando duas técnicas em conjunto: crioconcentração progressiva como sendo um primeiro estágio do processo, e crioconcentração em bloco assistida por vácuo como sendo o segundo estágio. A condição otimizada para o primeiro estágio foi encontrada utilizando uma temperatura do líquido refrigerante a – 5 ºC, agitação mecânica de 1035 rpm, e tempo de ensaio de 58 min. Escolhida essa condição (na qual têm-se como produtos as frações gelo e concentrado), o segundo estágio foi aplicado no gelo. Assim, a fração concentrada obtida ao

final de todo o processo continha teores de 6.7 g 100 g^{-1} e 10.24 g 100 g^{-1} de proteína e carboidrato, respectivamente. Finalmente, acredita-se que este trabalho é inovador quanto à principal matéria-prima (leite sem lactose), e quanto à utilização desta matéria-prima em processos de microencapsulação e crioconcentração. O leite é um alimento rico e de alto valor nutritivo; assim, o constante estudo desse alimento auxilia na criação de produtos inovadores e que atendam às demandas/exigências da sociedade.

Palavras-chave: Leite sem lactose. *Bifidobacterium* BB-12. Simulação das condições gastrointestinais. Análise térmica. Isoterma de adsorção. Alimento Funcional. *Skyr*. Textura. Proteína do leite. Concentrado. Crioconcentração em bloco.

ABSTRACT

It is estimated that lactose intolerance (whether temporary or permanent) afflicts a considerable part of the world population. The numbers reported in the literature vary between 65 and 75%. Seeking to create alternatives for this demand, as well as simply offering new possibilities for those who do not wish to consume lactose, this work investigated the potential of lactose-free milk in emerging and innovative technologies. In this sense, the experimental work was carried out in three major steps: First, lactose-free skimmed milk was used as an encapsulation matrix for a probiotic bacteria (*Bifidobacterium* BB-12). Not only milk, but also prebiotic carbohydrates (oligofructose and inulin) were used in the microencapsulation process by spray drying. The powders obtained were subjected to analyzes of encapsulation yield, microbial viability during storage (refrigeration temperature and room temperature), and probiotic survival during passage through the gastrointestinal system. In a second moment, the powders were also characterized in terms of their physicochemical properties. For this, extensive evaluations were made, including: study of color; determination of moisture, water activity and density; investigation of water sorption properties; thermal analysis; and morphology of the powders. The highest encapsulation yield (88.01%) and the highest viability of bifidobacteria during 120 days of storage at 25 °C were observed for the powder from lactose-free milk and inulin. On the other hand, the powder consisting of lactose-free milk and oligofructose showed the best survival of the probiotic after undergoing *in vitro* simulated gastrointestinal conditions. Likewise, when evaluating all the physicochemical properties of the powders, it was observed that the formulations containing the prebiotics were the ones that resulted in the most desirable characteristics of thermal stability, moisture and water activity. However, it was not concluded that the powder consisting only of lactose-free milk had unsatisfactory and/or unacceptable properties. For example, under refrigeration temperatures, microbial stability was also achieved for 120 days of storage. Also, when evaluating the thermal properties, it was found that this powder was stable up to ∼130 °C. Regarding water sorption, its characteristics were similar to those of powder obtained from milk and inulin; that is, relative humidity conditions above 33% are not indicated to maintain the physicochemical stability of these powders throughout storage. Because of this set of information, the probiotic powder based on lactose-free milk was chosen (as well as the others) to be studied in the second part of this thesis: application of these microparticles in a lactose-free dairy product. This product was developed in order to obtain a *Skyr*-style fermented milk, which is a typical food from Iceland. It is a concentrated fermented milk and therefore has a high protein content and is fat-free. Thus, four concentrated fermented milk formulations were prepared: three of them containing each probiotic powder, and a formulation containing bifidobacteria in the free state (called control sample). As expected, the inclusion of powders in fermented milk changed some properties, such as pH, acidity, and firmness. Because of these characteristics, the products added of the probiotic powders were reported as the best formulations for the development of a new lactose-free concentrated probiotic yogurt. The third and last part worked in this thesis was the use of freeze concentration in lactose-free skimmed milk. It was a stage carried out in partnership with the Universitat Politècnica de Catalunya (Spain). Milk freeze concentration was investigated using two techniques together: progressive freeze concentration as the first stage of the process, and vacuum-assisted block freeze concentration as the second stage. The optimized condition for the first stage was found using a coolant temperature at –5 °C, mechanical agitation of 1035 rpm, and an assay time of 58 min. After choosing this condition (in which the ice and concentrate fractions are the products), the second stage was applied to ice. Thus, the concentrated fraction obtained at the end of the entire process had contents of 6.7 g 100 g^{-1} and 10.24 g 100 g^{-1} of protein and carbohydrate, respectively. Finally, it is believed that this work

is innovative in terms of the main raw material (lactose-free milk), and in terms of the use of this raw material in microencapsulation and freeze concentration processes. Milk is a rich food with high nutritional value; thus, the constant study of this food helps in the creation of innovative products that meet the demands/requirements of society.

Keywords: Lactose-free milk. *Bifidobacterium* BB-12. Simulation of gastrointestinal conditions. Thermal analysis. Adsorption isotherm. Functional food. *Skyr*. Texture. Milk protein. Concentrate. Block freeze concentration.

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1 INTRODUÇÃO

Os microrganismos probióticos são constantemente estudados devido aos seus efeitos benéficos na saúde humana, como a contribuição para o equilíbrio da microbiota intestinal, dando suporte para os sistemas digestório e imunológico. Nesse contexto, o mercado de probióticos movimentou 48,88 bilhões de dólares em 2019 e há perspectiva de aumento para 94,48 bilhões até 2027. Essa expansão se deve à ciência do consumidor a respeito dos benefícios à saúde, além da demanda por produtos que aumentam a imunidade frente a pandemia da COVID-19 (MARKET RESEARCH REPORT, 2020).

Bactérias probióticas foram incorporadas com sucesso a uma ampla gama de alimentos, dentre eles, leite e produtos lácteos (BLAIOTTA *et al*.; 2017; VALENCIA *et al*.; 2016; VERRUCK *et al*.; 2015). No entanto, um segmento significativo da população mundial adulta (aproximadamente 70%) apresenta intolerância permanente ou temporária à lactose (UGIDOS-RODRÍGUEZ; MATALLANA-GONZÁLEZ; SÁNCHEZ-MATA, 2018). No caso da população africana e sul-americana, estima-se que 50% das pessoas sejam afetadas (DE OLIVEIRA NEVES; LEAL DE OLIVEIRA, 2021). A prevalência dessa incapacidade de digerir lactose pode estar associada à redução geneticamente programada da atividade da lactase durante a vida adulta. Em caso de consumo de lácteos que contenham lactose, os indivíduos podem apresentar distúrbios digestivos graves (CORGNEAU *et al*., 2017). Assim, uma das alternativas que busca solucionar esse problema é o consumo de leite sem lactose (DEKKER; DAAMEN, 2011). Embora exista um grande número de derivados lácteos probióticos disponíveis no mercado, há uma carência de lácteos probióticos sem lactose. Dado o alto valor nutritivo dos laticínios e a relevância das bactérias probióticas, o desenvolvimento de produtos que atendam a esses requisitos é de grande valia.

A microencapsulação de probióticos se constitui hoje numa tecnologia amplamente difundida e aceita, pois isso daria às bactérias mais proteção e/ou estabilidade durante a passagem pelo trato gastrointestinal. De acordo com Dianawati *et al*. (2017), a secagem por *spray drying* é o método mais comum de microencapsulação para produzir probióticos desidratados. O custo relativamente baixo e a facilidade de operação e ampliação de escala são algumas das vantagens desse método (GHANDI *et al*., 2012). A viabilidade das células após o processo de *spray drying* depende de alguns fatores, dentre eles, os agentes de encapsulação (FRITZEN-FREIRE *et al*., 2012). São listados diferentes tipos de agentes na literatura, incluindo polissacarídeos. Os polissacarídeos com propriedades prebióticas, como inulina e

oligofrutose, têm sido utilizados para proteger bactérias probióticas durante a secagem por *spray drying* e em condições de armazenamento (FRITZEN-FREIRE *et al*., 2013; PINTO *et al*., 2015). Em suma, a adição de prebióticos em conjunto com alimentos probióticos é interessante devido ao seu efeito simbiótico e, frequentemente, termoprotetor.

Outra tecnologia que vem ganhando espaço no estudo dos lácteos é a crioconcentração. Segundo Aider e Halleux (2009), se trata de uma técnica aplicada principalmente na elaboração de ingredientes e alimentos de alto valor nutritivo. Nesse processo, a concentração do fluido ocorre por meio da retirada de água na forma de cristais de gelo. Isso significa que a temperatura da solução de interesse é reduzida para abaixo do seu ponto de congelamento (GULFO *et al*., 2014), com o objetivo de evitar a temperatura eutética e consequentemente a solidificação de todos os constituintes do produto. Uma das principais vantagens dessa operação é a preservação de compostos termicamente sensíveis, dadas as baixas temperaturas de processamento empregadas (De LIZ *et al*., 2020). O tratamento do leite em uma faixa de temperatura de 70 a 100 °C pode desnaturar a proteína do soro de leite (por exemplo, α-lactalbumina e β-lactoglobulina) e induzir a formação de agregados (QIAN *et al*., 2017). Além disso, Amran *et al*. (2016) discutiram que a evaporação (operação unitária comumente usada para leite) requer uma quantidade de energia de 2260 kJ/kg para remover a água. Em contraste, Jusoh *et al*. (2008) comentaram que para o processo de crioconcentração, a energia necessária é de aproximadamente 335 kJ/kg de água. Analisando por essa via, sugere-se então que a crioconcentração possibilita remover a água de maneira mais seletiva do que outras técnicas tradicionais (YEE; WILEY; BAO, 2007), e além disso, com custos menores (SÁNCHEZ *et al*., 2011).

Diante dessas motivações e relevância, este estudo foi conduzido para explorar o potencial do leite sem lactose como agente encapsulante de bactéria probiótica, e ainda verificar a aplicabilidade desse composto em uma matriz láctea consideravelmente consumida (iogurte concentrado). Levando em conta que o leite é constituído por ≈ 88 g 100 g^{-1} de água, caso o mesmo esteja concentrado, muitos processos envolvidos na produção de derivados poderiam ser otimizados, o que aumentaria as propriedades nutricionais e tecnológicas do produto obtido. Portanto, este trabalho também objetivou investigar a crioconcentração do leite sem lactose, aplicando as técnicas de crioconcentração progressiva e crioconcentração em bloco assistida por vácuo. Assim, esta tese segue uma estrutura de apresentação das informações, que está detalhadamente explicada no subitem seguinte.

1.1 ESTRUTURA DO TRABALHO

Este trabalho foi estruturado em uma seção e 5 capítulos, seguindo uma sequência lógica baseada nas suas etapas de execução. Assim, encontra-se ordenado da seguinte forma:

Seção 1. Introdução

Abordagem geral sobre o quadro da intolerância à lactose, consumo de lácteos sem lactose, microrganismos probióticos, e produto sem lactose funcional. Também foi explorada a crioconcentração, uma técnica que visa agregar valor a alimentos líquidos, mas que também pode ser empregada no tratamento de resíduos. Ademais, foram apresentados os objetivos gerais da tese.

Capítulo 1. Revisão bibliográfica

Este capítulo discute o cenário global da intolerância à lactose, além de uma descrição sobre as mais relevantes inovações em leites e produtos lácteos sem lactose.

São apresentados os conceitos existentes para microrganismos probióticos e substâncias prebióticas. Suas definições, funções e utilizações gerais foram abordadas. Além disso, foi relatado o efeito da aplicação dos probióticos, prebióticos ou sua associação (simbióticos) em leite e derivados lácteos.

Discutimos também as variadas formas de encapsular microrganismos probióticos, uso de probióticos microencapsulados em alimentos, agentes encapsulantes empregados neste trabalho, e importância da caracterização dos pós obtidos.

Por fim, abordamos sucintamente, a técnica de crioconcentração, os diferentes mecanismos de formação dos cristais, e sua aplicação em alimentos.

Capítulo 2. Lactose-free skim milk and prebiotics as carrier agents of *Bifidobacterium* BB-12 microencapsulation: physicochemical properties, survival during storage and *in vitro* gastrointestinal condition behaviour

O objetivo desta parte experimental foi avaliar o efeito da microencapsulação por *spray drying* na sobrevivência de *Bifidobacterium* BB-12 ao usar como agentes encapsulantes leite desnatado sem lactose, leite desnatado sem lactose e inulina, e leite desnatado sem lactose e oligofrutose. Bactéria livre e microencapsulada foram submetidas à simulação do sistema gastrointestinal *in vitro*. Este trabalho foi publicado. DOI: **https://doi.org/10.1111/ijfs.14823**

Capítulo 3. Current knowledge about physical properties of innovative probiotic spray-dried powders produced with lactose-free milk and prebiotics

Os pós resultantes do processo de microencapsulação foram submetidos a análises físicas e morfológicas (microscopia eletrônica de varredura, termogravimetria, calorimetria exploratória diferencial, espectroscopia raman e difração de raios X). As propriedades de adsorção de água também foram estudadas por meio de cinéticas de equilíbrio. Este trabalho foi publicado. DOI: **https://doi.org/10.1016/j.lwt.2021.112175**

Capítulo 4. Encapsulated *Bifidobacterium* BB-12 addition in a concentrated lactose-free yogurt: its survival during storage and effects on the product's properties

Um iogurte concentrado do tipo *skyr* (leite fermentado concentrado tradicional da Islândia) foi investigado como matriz para incorporação de *Bifidobacterium* BB-12 microencapsulado. Para tal, foi utilizado leite sem lactose nas formulações dos iogurtes, visando garantir a ausência desse dissacarídeo, já que para uma parcela da população, a presença de pequenas quantidades já traria desconfortos abdominais. Este trabalho encontrava-se em processo de publicação durante o depósito desta tese.

Capítulo 5. The combined use of progressive and block freeze concentration in lactose-free milk: effect of process parameters and influence on the content of carbohydrates and proteins

Estudou-se o comportamento de leite desnatado sem lactose nos processos de crioconcentração progressiva e crioconcentração em bloco assistida por vácuo. Este trabalho foi realizado na Universitat Politècnica de Catalunya (UPC), Barcelona. Os recursos foram provenientes do projeto PrInt – CAPES número 88887.310560/2018-00. Este trabalho foi publicado. DOI: **https://doi.org/10.1111/jfpe.13867**

Outros estudos foram desenvolvidos junto ao grupo de pesquisa, incluindo trabalhos com soro, leite de cabra, e crioconcentração. Alguns estão relatados no Anexo B, assim como trabalhos apresentados em eventos científicos (Anexo A).

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CAPÍTULO 1

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1 LEITE E INTOLERÂNCIA À LACTOSE

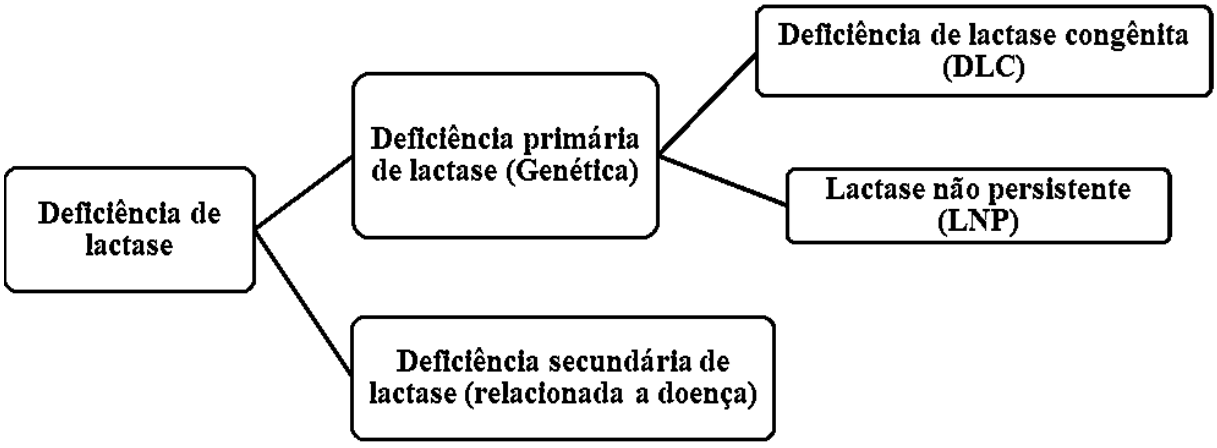
O leite, sem outra especificação, é definido como o produto oriundo da ordenha completa e ininterrupta, em condições de higiene, de vacas sadias, bem alimentadas e descansadas (BRASIL, 2011). O Brasil detém a quarta colocação entre os produtores mundiais de leite e a primeira entre os países da América do Sul. O leite encontra-se ainda entre os seis produtos mais importantes da agropecuária brasileira, à frente inclusive de *commodities* tradicionais como café e suco de laranja (FAOSTAT, 2017). Desta forma, no Brasil, o leite desempenha papel relevante para a população como alimento, gerando emprego e contribuindo para a renda (COSTA; QUEIROGA; PEREIRA, 2009). Entretanto, apesar desta produção, o Brasil ainda é um grande importador de produtos lácteos (EPAGRI, 2017). Cabe ressaltar que a importação destes produtos ocorre devido às exigências de consumidores, que consequentemente irão pautar mudanças na estratégia de pesquisas e produção. Sendo assim, para suprir a demanda interna de produtos, ainda há muito potencial para a produção leiteira no Brasil, principalmente no que diz respeito à produção de derivados lácteos sem lactose.

O consumo de leite UHT sem lactose aumentou 224% no Brasil desde 2014, e representa 4% do mercado de laticínios. As vendas de produtos lácteos sem lactose superam os US$ 300 milhões e US$ 2 per capita anuais. Além disso, apresenta uma tendência de alta anual estimada entre 10% e 15% nos próximos cinco anos (MILKPOINT, 2018). Esse aumento no consumo de produtos lácteos sem lactose está relacionado com a crescente ocorrência de intolerância à lactose ou a dietas especiais. A intolerância à lactose se caracteriza pela diminuição ou ausência da produção da enzima lactase-florizina hidrolase (NP_002290.2- número de acesso no NCBI), que tem a função de hidrolisar a lactose em glicose e galactose, e assim serem absorvidas no intestino delgado. A lactase-florizina hidrolase é, portanto, essencial para a nutrição de mamíferos recém-nascidos, cuja única fonte de nutrição é o leite (SWALLOW, 2011). Essa enzima é codificada pelo gene *LCT* (NG_008104 - número de acesso no NCBI), e verificou-se que a capacidade de digerir a lactose na idade adulta é devido às mutações *cis-acting* na expressão gênica, que são herdadas de forma dominante. A lactose, ao contrário dos seus monossacarídeos (isto é, glicose e galactose), é pouco absorvida no intestino delgado. Portanto, as pessoas que não carregam mutações no gene *LCT* não digerem e absorvem a lactose, o que levará ao quadro clínico de intolerância à lactose (DEKKER; DAAMEN, 2011).

A deficiência de lactase pode ser genética (deficiência primária de lactase) ou relacionada à doença (deficiência secundária de lactase) (Figura 1). A deficiência primária de lactase pode se apresentar de duas maneiras diferentes. A mais grave é conhecida como

deficiência de lactase congênita (DLC), e é uma doença autossômica recessiva rara que afeta recém-nascidos (BERG *et al*., 1969). O recém-nascido apresenta diarreia líquida ao ser amamentado ou receber fórmulas contendo lactose, e caso não seja diagnosticada precocemente, a DLC pode levar ao óbito devido à desidratação intensa (MATTAR; MAZO, 2010).

Figura 1 – Tipos de deficiencia de lactase.



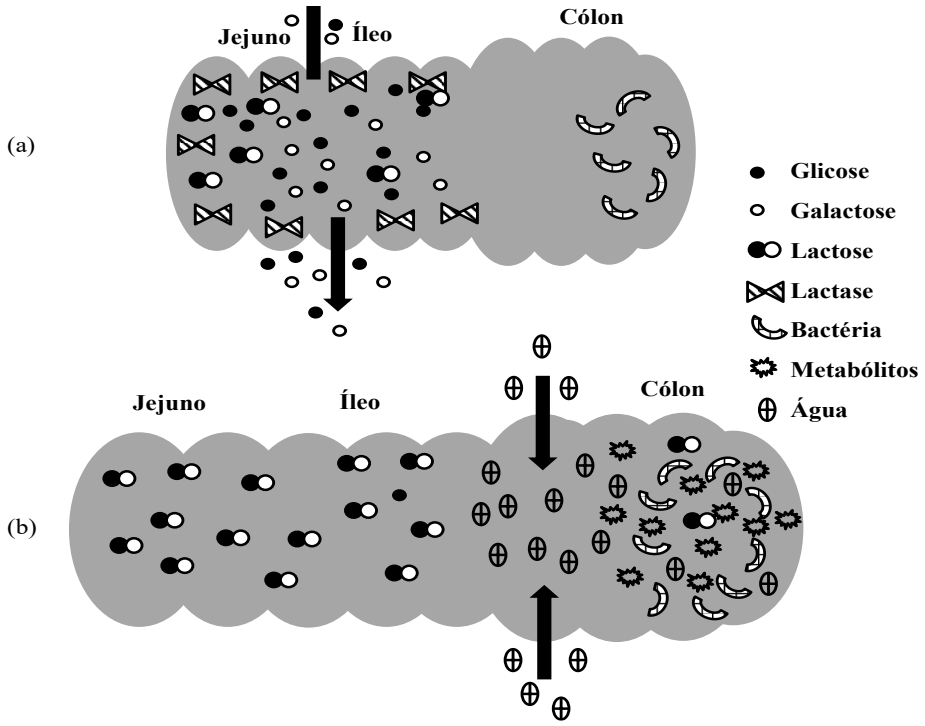
Fonte: Adaptado de Corgneau *et al*. (2017).

Kuokkanen *et al*. (2006) relataram cinco mutações distintas na região codificadora do gene da lactase (*LCT*) em pacientes com DLC. Em outras palavras, a DLC representa o resultado de mutações que afetam a estrutura da proteína, com consequente inativação da enzima lactase-florizina hidrolase. Esse cenário é diferente da forma mais comum de intolerância à lactose (hipolactasia primária do adulto), que por sua vez é causada por uma variante do elemento regulador. Estas descobertas facilitaram o teste genético na prática clínica e permitiram o aconselhamento genético para a DLC. Para os portadores desta doença, a dieta restritiva de lactose é necessária para o desenvolvimento normal do recém-nascido (ROBAYO-TORRES; NICHOLS, 2007).

A deficiência de lactase não persistente (LNP), também chamada de hipolactasia primária do adulto, é a forma menos grave de intolerância à lactose, e consiste numa regulação negativa da atividade da lactase em células intestinais após o desmame (MATTAR; MAZO, 2010). Conforme pode ser visualizada na Figura 2a, a conversão de lactose em glicose e galactose requer atividade de lactase. A lactose-florizina hidrolase intestinal possui dois sítios ativos: um catalisa a hidrólise da lactose, enquanto o outro catalisa a hidrólise de outros carboidratos (incluindo a florizina). A lactose-florizina hidrolase está localizada no intestino

delgado e apresenta maior expressão no jejuno (CORGNEAU *et al*., 2017). Assim como outras enzimas envolvidas na digestão e absorção de carboidratos, a lactose-florizina hidrolase está ancorada na superfície da borda em escova (SWALLOW, 2011). Em condições normais, após a hidrólise da lactose pela lactose-florizina hidrolase, a glicose e a galactose são transportadas através das membranas celulares epiteliais para os enterócitos e depois para a corrente sanguínea (WRIGHT; HIRAYAMA; LOO, 2007). A glicose entra para o pool de glicose do intestino, e a galactose é metabolizada no fígado para ser convertida em glicose, entrar nesse pool e servir como fonte de energia (MATTAR; MAZO, 2010). Em caso de deficiência de lactase, a lactose ingerida não é degradada no intestino delgado e passa para o cólon, onde serve como fonte de energia para a abundante microbiota (Figura 2b).

Figura 2 – Representações esquemáticas (a) da digestão normal da lactose, e (b) da digestão quando há deficiência de lactase.



Fonte: A autora.

Entre as centenas de bactérias do cólon, algumas têm a capacidade de metabolizar a lactose, em particular as bifidobactérias, lactobacilos e *Escherichia coli*. As β-galactosidases bacterianas catalisam as mesmas reações químicas que a lactose-florizina hidrolase, mas diferem desta na estrutura, nas propriedades enzimáticas e na regulação (CORGNEAU *et al*., 2017). Quando as β-galactosidases bacterianas liberam glicose e galactose, as bactérias intestinais as convertem em uma variedade de produtos, incluindo ácidos graxos de cadeia curta

(AGCCs) e gás hidrogênio (CORGNEAU *et al*., 2017). Essa fermentação geralmente causa dor abdominal e inchaço. O fato de a lactose não ser hidrolisada em glicose e galactose impede o aumento da concentração de glicose no sangue que é normalmente observada após a ingestão de lactose. Assim, a má absorção de lactose pode ser verificada por uma análise de glicose no sangue. Uma concentração de glicose no sangue que aumenta menos de 20 mg/100 mL após a ingestão de uma grande dose de lactose (de 50 a 100 g) é indicativa de deficiência de lactase (LEVITT; WILT; SHAUKAT, 2013).

A presença de produtos de fermentação no cólon aumenta a pressão osmótica no lúmen. Devido à alta permeabilidade hidráulica da mucosa do cólon, o intestino não consegue manter um bom gradiente osmótico entre o sangue e o lúmen, de modo que a água se move do sangue para o lúmen para tornar os conteúdos luminais isotônicos (CORGNEAU *et al*., 2017). Dependendo da quantidade de lactose no cólon, o influxo de água pode causar severas diarreias e considerável perda de líquido (LEVITT; WILT; SHAUKAT, 2013). Os AGCCs não parecem causar problemas, pois são absorvidos pelo sangue e servem como fonte de energia. Mas, além disso, a quantidade de gás produzida pelas reações de fermentação bacteriana pode ser enorme. A degradação de 12,5 g de lactose (equivalente a aproximadamente um copo de 250 mL de leite fluido) pode liberar até 2600 mL de CO_2 e 4000 mL de H_2 (WOLIN, 1981), enquanto a taxa de excreção usual é inferior a 1000 mL de gás por dia (TOMLIN; LOWIS; READ, 1991). Uma grande parte desses gases é excretada, causando flatulência, enquanto o restante é absorvido pelo intestino e expirado pelos pulmões com a respiração. O H_2 exalado é hoje utilizado como diagnóstico não invasivo de intolerância à lactose. É comumente considerada a técnica mais confiável, não invasiva e barata (LOMER; PARKES; SANDERSON, 2007). Uma concentração de H_2 superior a 20 ppm na expiração acima da linha de base indica intolerância à lactose (JÄRVELÄ; TORNIAINEN; KOLHO, 2009). Os sintomas clínicos aparecem entre 1 e 3 h após o consumo e é intimamente dependente da quantidade de lactose ingerida, com grandes variações entre os indivíduos (EFSA, 2010). Portanto, a diferença entre a hipolactasia primária do adulto (LNP) e a deficiência de lactase congênita (DLC) é molecular: na primeira, a enzima lactase é normal, mas diminui a expressão ao longo da vida; na segunda, a enzima lactase está ausente ou é truncada (ROBAYO-TORRES; NICHOLS, 2007).

A deficiência secundária de lactase é causada por doenças ou tratamentos que lesionam a mucosa intestinal (Doença de Crohn, inflamação intestinal crônica, quimioterapia para câncer, etc.) (MATTAR; MAZO, 2010). Uma vez que a enzima lactase-florizina hidrolase localiza-se na borda em escova da mucosa, qualquer alteração morfológica poderá impactar na diminuição da capacidade de hidrolisar a lactose (OLIVEIRA, 2013). Mesmo que a lactase pareça ser a

enzima mais vulnerável, as atividades de outras dissacaridases e enzimas de borda em escova também são reduzidas (FLATZ, 1987). Esta condição é apenas temporária e a atividade da lactase reaparece uma vez que o epitélio se recupera (EFSA, 2010).

A prevalência da LNP está fortemente relacionada à etnia (WELSH *et al*., 1978). Em geral, a grande maioria dos indivíduos que se originam de zonas onde a ordenha não é tradicional, são maus digestores de lactose (70 – 100%). Por outro lado, indivíduos provenientes de locais com uma antiga tradição de consumo de leite, apresentam baixa prevalência de má digestão de lactose (CORGNEAU *et al*., 2017). A Tabela 1 apresenta a prevalência de intolerância à lactose em adultos de diferentes países. Com exceção da população da Europa setentrional e central (e de seus descendentes nas Américas e na Australásia), cerca de 75% da população mundial é constituída por indivíduos lactase não persistentes, dos quais 30% são assintomáticos. Isso significa que 50% da população adulta mundial sofre de intolerância à lactose. A prevalência de má digestão é acima de 50% na América do Sul e na África, e chega a quase 100% em alguns países asiáticos. Nos Estados Unidos, a prevalência varia de 15 (em caucasianos) a 80% (em populações afro-americanas). Na Europa, há pequenas variações: aproximadamente 2% da população na Escandinávia é intolerante, 17% na Finlândia, e na Itália, 2 a 70% (DEKKER; DAAMEN, 2011). Portanto, leite e muitos outros produtos lácteos não fazem parte da dieta regular de grande parte da população mundial. Visto que o leite se constitui numa fonte de nutrientes essenciais (como proteína, riboflavina, cálcio, vitamina D, niacina, vitamina B12, fósforo, magnésio, vitamina A, zinco e iodo), a não ingestão de laticínios poderia ser associada à deficiências nutricionais (DEKKER; DAAMEN, 2011).

Tabela 1 – Prevalência da intolerância à lactose em adultos de diferentes países.

Local	**Prevalência (%)**
Alemanha	14,8
Áustria	20,1
Brasil (brancos)	57
Brasil (crianças índias Terenas)	89,3 após 4 anos
Brasil (japoneses)	100
Brasil (mulatos)	57
Brasil (negros)	80
China (Norte)	87,3
Estônia (ligados aos finlandeses)	24,8

Local	Prevalência (%)
França	23,4
Hungria	37
Índia (Norte)	67,5
Índia (sul)	86,8
Itália	51(Norte) 71(Sicilia)
Japão (adultos)	89
Jordânia (beduínos)	24
Jordânia (oeste) e Palestina	75
Rússia (Nordeste)	35,6
Sibéria (oeste, nativos Khants)	94
Somalis	76
Sudão (tribo Béja, pecuaristas)	16,8
Sudão (tribo Nilotes, agricultores)	74,5
Suécia (crianças caucasianas)	10
Suécia (crianças não-caucasianas)	66
Suécia (idoso caucasiano)	5
Tuaregues	12,7
Turquia	71,3

Fonte: Adaptado de Mattar e Mazo (2010).

É importante salientar que há diferenças significativas entre a intolerância à lactose e a alergia à proteína do leite de vaca (APLV). O Quadro 1 exemplifica resumidamente as principais diferenças entre elas. Algumas pessoas intolerantes à lactose ainda apresentam a percepção de que é preciso retirar o leite e os derivados lácteos da sua dieta. A retirada se justifica apenas quando quadros alérgicos estão presentes (APLV), e se a deficiência de lactase-florizina hidrolase é congênita, ou ainda se houver um diagnóstico de galactosemia (patologia caracterizada pela inabilidade do organismo de converter galactose em glicose). Nos casos de hipolactasia primária do adulto, a retirada do leite e derivados da dieta não é necessária e também não recomendada (FAO, 2013). A recomendação diária de consumo de leite e derivados lácteos varia entre os países e faixa etária do indivíduo. De acordo com a Organização das Nações Unidas para Agricultura e Alimentação (FAO), essa recomendação é de uma (1)

porção até cinco (5), correspondendo a quantidades que variam entre 250 a 800 mL de leite fluido (FAO, 2013).

Quadro 1 – Comparação entre Alergia ao Leite de Vaca (APLV) e Intolerância à Lactose.

	Alergia à Proteína do Leite	**Intolerância à lactose**
Causa	Resposta imune anormal à ingestão de proteína do leite de vaca.	Baixos níveis intestinais da enzima lactase que digere a lactose (açúcar do leite).
Idade de início	Geralmente na infância	Infância precoce/tardia
Sintomas	Dor abdominal, vômitos, diarreia, congestão nasal, erupção cutânea, ...	Produção de gases no abdômen, inchaço, cólicas, diarreia, ...
Diagnóstico	Eliminação de alimentos e testes; Teste de sangue RAST	Teste de hidrogênio na respiração, teste genético ou teste de curva de glicose
Uso/Prevenção de leite e derivados	Eliminar a proteína do leite de vaca da dieta	Não há necessidade de eliminar alimentos lácteos; experimentar diferentes quantidades/tipos de alimentos lácteos para melhorar a tolerância; uso de produtos lácteos comerciais sem lactose ou de baixo teor de lactose.

Fonte: Adaptado de Miller *et al*. (2000).

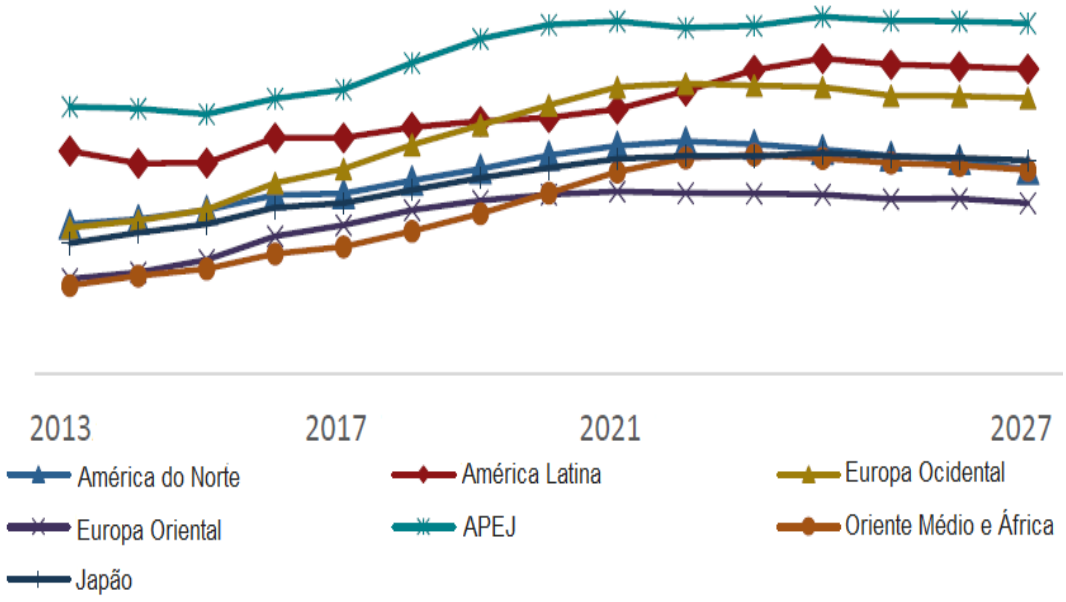
Como já mencionado, as pessoas que consomem menos leite e outros alimentos lácteos por causa da intolerância à lactose geralmente têm menor ingestão de cálcio e outros nutrientes fornecidos pelo leite. Uma ingestão inadequada de cálcio aumenta o risco de osteoporose, hipertensão e, possivelmente, de câncer de cólon (MILLER *et al*., 2000). O grau de má absorção de lactose varia muito entre os seres humanos, mas a maioria pode ingerir até 18 g de lactose diariamente sem apresentar nenhum sintoma (CORGNEAU *et al*., 2017). A gravidade dos sintomas depende muito da taxa de esvaziamento gástrico, do tempo de trânsito intestinal, e da microbiota ali presente. Portanto, a constituição da refeição ingerida junto com a lactose é importante. Em geral, a ingestão de lactose através de um produto de maior viscosidade, como o iogurte, resultará em menos problemas se comparada ao consumo de um produto lácteo líquido (DEKKER; DAAMEN, 2011).

A pesquisa sobre os fatores envolvidos na digestão da lactose estimulou o desenvolvimento de estratégias que permitem que pessoas com baixa atividade de lactase consumam produtos lácteos sem apresentar sintomas desagradáveis (MILLER *et al*., 2000). Hoje em dia, mais e mais produtos lácteos de baixo teor de lactose estão comercialmente disponíveis. O uso desses produtos ou produtos isentos de lactose alivia os problemas associados à digestão da lactose. Dessa forma, uma grande parte da população mundial pode se beneficiar dos nutrientes essenciais presentes nos lácteos (DEKKER; DAAMEN, 2011).

1.1 INOVAÇÃO EM LEITES E PRODUTOS LÁCTEOS SEM LACTOSE

Segundo um relatório de pesquisa publicado em 2019 pela FMI (Future Market Insights, fornecedora de serviços de consultoria e inteligência de mercado, sediada em Londres), a demanda global por alimentos deverá crescer de 59% para 98% nos próximos 30 anos, sendo cada vez mais importante para os fabricantes de produtos alimentícios introduzir alimentos inovadores em mercados já consolidados, como o de produtos lácteos sem lactose. A pesquisa revela ainda que a região do mundo em que há maior valor de mercado para os produtos lácteos sem lactose é a APEJ (Ásia-Pacífico, exceto Japão) (Figura 3), além de aumento e valorização desses produtos também na América Latina.

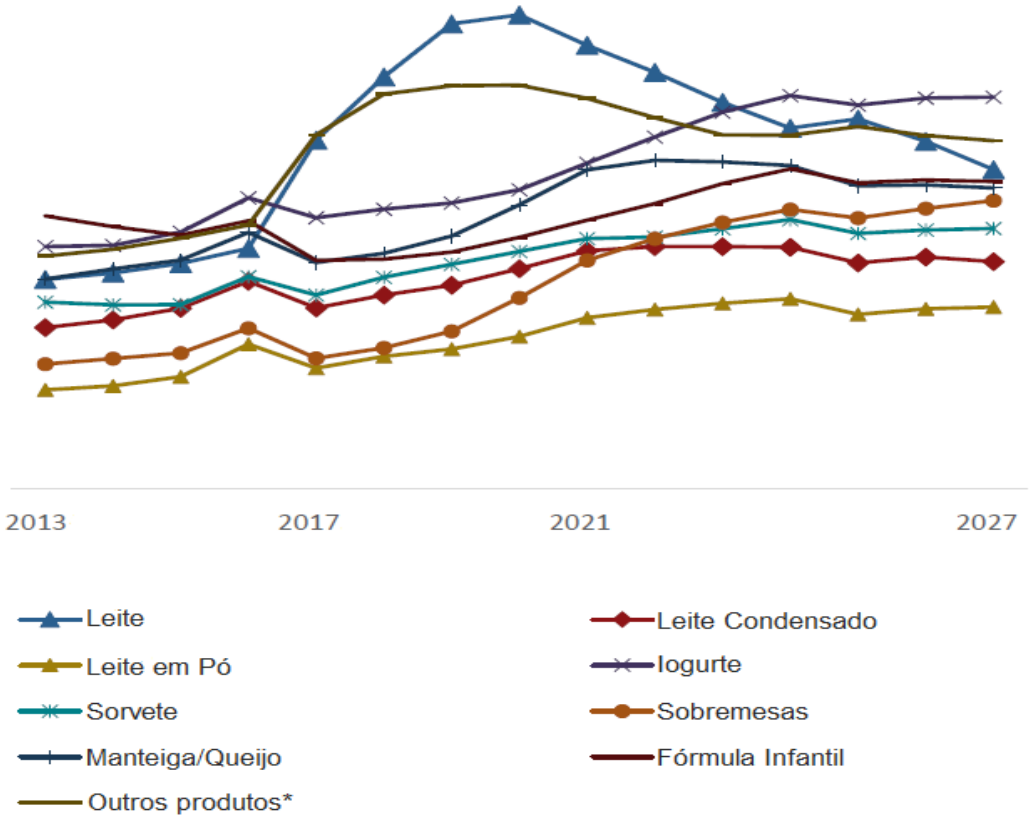
Figura 3 – Crescimento global ano após ano do valor de mercado de produtos lácteos sem lactose, por região (2017–2027).



Fonte: Adaptado de FMI (2018).

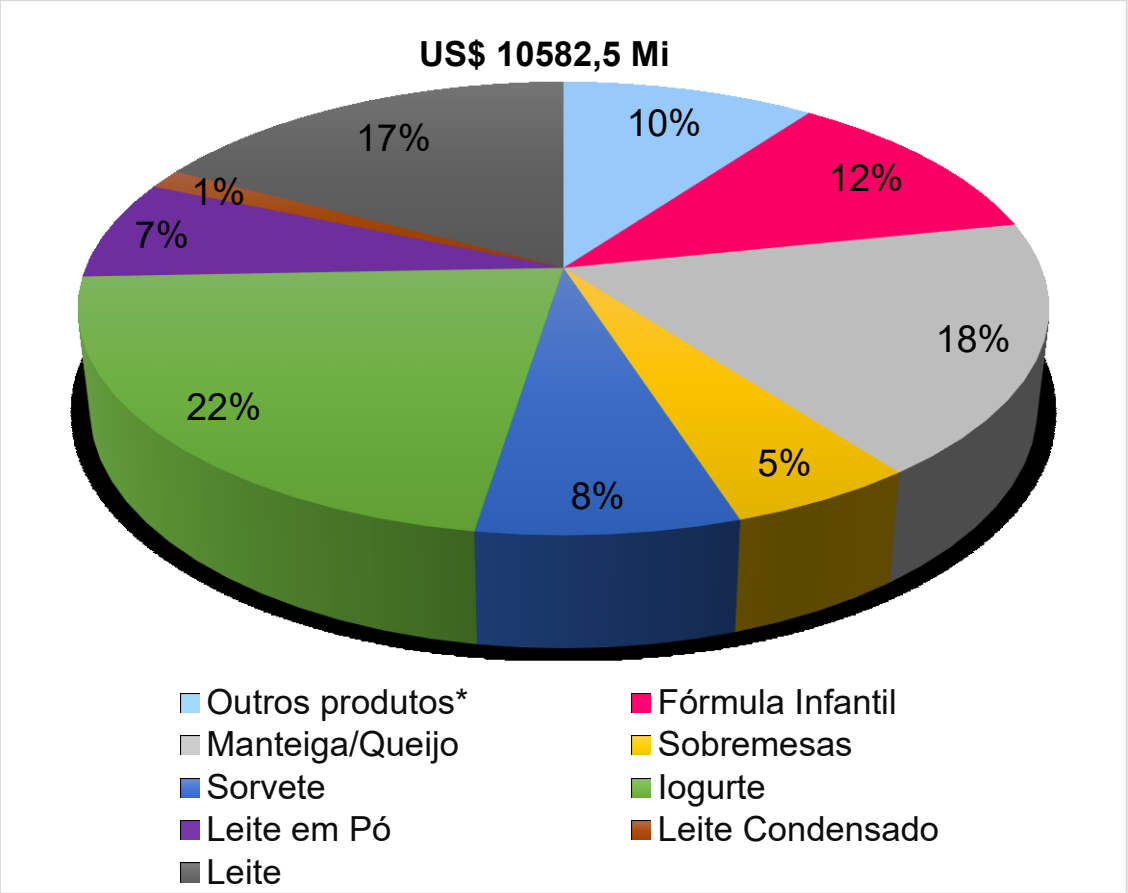
Em relação aos diversos tipos de produtos lácteos, observa-se uma tendência de queda do valor de mercado do leite sem lactose (Figura 4), se opondo ao valor de mercado do iogurte sem lactose, que tende a aumentar até 2024. Em 2017 já se percebeu que a maior parte da movimentação financeira global dos lácteos sem lactose veio do iogurte (Figura 5), representando 21,9% do valor de mercado, seguido do queijo e manteiga (18%). Apesar das tecnologias para produzir leite com lactose reduzida, mudanças na demografia e estilo de vida nos Estados Unidos não favoreceram o crescimento do consumo de leite fluido (BARBANO, 2017). Recentemente, uma combinação de técnicas (remoção parcial de lactose por ultrafiltração e hidrólise enzimática da lactose remanescente) produziu um leite fluido reduzido em calorias e lactose (Fairlife, Chicago, IL). O Quadro 2 resume todos os estudos de produtos lácteos inovadores, apresentados e discutidos detalhadamente nos parágrafos abaixo.

Figura 4 – Crescimento global ano após ano do valor de mercado de produtos lácteos sem lactose, por tipo de produto (2017–2027).



Fonte: Adaptado de FMI (2018).

Figura 5 – Análise BPS global do valor de mercado de produtos lácteos sem lactose, por tipo de produto (2017).



Fonte: Adaptado de FMI (2018). (*)Preparações culinárias, sobremesas regionais, kefir, produtos de confeitaria e chocolates; com teor de lactose zero ou reduzido.

Quadro 2 – Produtos lácteos com baixo teor de lactose ou sem lactose desenvolvidos recentemente.

Produto	**Tratamento prévio da matéria-prima**	**Teor de lactose no(s) produto(os) final(is)**	**Autores**
Leite prebiótico	Transgalactosilação da lactose	2,1 g/L	(PLOU *et al*., 2016)
Iogurte prebiótico	Transgalactosilação da lactose	1,44%	(RAZA *et al*., 2018)
Iogurte probiótico	Hidrólise da lactose	<LDM	(VARGA; ROMAN; TOTH, 2004)
Iogurte	Hidrólise da lactose	≤0,01 mg / 100 cm^3	(TONGUC; KARAGOZLU, 2017)
Iogurte adicionado de cranberry e mirtilo	Hidrólise da lactose	≤0,01%	(DABIJA; ROPCIUC, 2016)
Bebida fermentada à base de soro	Hidrólise da lactose	-	(MATIJEVIĆ; LISAK; BOŽANIĆ, 2011)
Bebida láctea com preparado de toranja	Ultrafiltração e hidrólise da lactose	<1%	(RAHIMI *et al*., 2017)
Queijo cottage prebiótico	Hidrólise da lactose	<0,1 g / 100 g	(NICOLETTI; VERDI; ENDRES, 2016)
Queijo muçarela	Ultrafiltração	0,00%	(MOYNIHAN *et al*., 2016)
Queijo Canestrato Pugliese	Adição de Bifidobacterium bifidum Bb02 (com consequente hidrólise da lactose)	<LDM	(CORBO *et al*., 2001)
Frozen	Hidrólise da lactose	<LDM	(SKRYPLONEK *et al*., 2017)
Achocolatado	Hidrólise da lactose	<LDM	(LI *et al*., 2015)

Fonte: A autora. LDM: valor abaixo do Limite de Detecção do Método.

Os alimentos funcionais têm o potencial de promover a saúde por meio de mecanismos não encontrados na nutrição convencional, e os efeitos restringem-se à promoção da saúde e bem-estar, maximizando as funções fisiológicas do indivíduo em vez de curar doenças (GRANATO *et al*., 2010). Aproximadamente 60% dos europeus consomem alimentos funcionais, de modo que as mulheres consomem a maior quantidade de bebidas funcionais (FMI, 2018). No Brasil, os alimentos com alegações de propriedades funcionais e ou de saúde são regulamentados pela Agência Nacional de Vigilância Sanitária. Ao todo, são seis grandes grupos que compõem a lista de nutrientes e não nutrientes que tem alegações padronizadas, e entre estes estão os microrganismos probióticos e alguns compostos alimentares com efeito prebiótico (ANVISA, 2002).

A literatura carece de pesquisas relacionadas à produção de derivados lácteos probióticos sem lactose, mas sabe-se que o consumo de produtos ricos em bactérias probióticas é proposto como estratégia de redução dos sintomas de intolerância a lactose, pois algumas delas têm a capacidade de metabolizar o dissacarídeo, como é o caso das bifidobactérias e lactobacilos (CORGNEAU *et al*., 2017). Isso foi observado nos trabalhos de Corbo *et al*. (2001) e Vesa *et al*. (1996). Vesa *et al*. (1996) estudaram o leite fermentado da marca Ofilus, que continha *Lactobacillus acidophilus* e *Bifidobacterium sp.*, e verificaram nos adultos voluntários com deficiência de lactase boa tolerância e digestibilidade da lactose. Corbo *et al*. (2001) analisaram as propriedades microbiológicas e bioquímicas do queijo Canestrato Pugliese suplementado com bifidobactérias (*Bifidobacterium bifidum* Bb02 e *Bifidobacterium longum* Bb46). Em contraste com o queijo controle (sem adição dos probióticos), a lactose foi completamente hidrolisada nos queijos elaborados com as bifidobactérias, e esses dados corroboram com as atividades encontradas para α- e β-galactosidase — que foram marcadamente mais pronunciadas na presença de bifidobactérias, especialmente *B. bifidum* Bb02.

Até o momento poucos produtos probióticos sem lactose à base de leite sem lactose (lactose previamente hidrolisada) foram documentados na literatura: Varga, Roman e Toth (2004) produziram iogurtes probióticos utilizando *Bifidobacterium bifidum*, *Lactobacillus helveticus* e *Lactobacillus acidophilus*. Pinto *et al*. (2019) incorporaram microcápsulas probióticas (*Bifidobacterium lactis* BB-12) em iogurte grego sem lactose. Matijević, Lisak e Božanić (2011) também elaboraram um derivado probiótico sem lactose, mas com outra matéria-prima: Whey Protein reconstituído e subsequentemente hidrolisado. Assim, foram desenvolvidas duas bebidas de soro fermentadas, uma contendo o probiótico *Lactobacillus acidophilus* La-5 e a outra com *Bifidobacterium animalis* subsp. *lactis* BB-12.

A β-galactosidase pode catalisar também a reação de transgalactosilação da lactose, produzindo consequentemente, prebióticos denominados galacto-oligossacarídeos (GalOS). Com base nesse conhecimento, Plou *et al*. (2016) desenvolveu um leite com presença significativa de GalOS e, ao mesmo tempo, baixo teor de lactose (concentração final de 2,1 g/L, sendo que inicialmente era 45 g/L). A justificativa desse trabalho se deu no fato de que os produtos lácteos infantis são geralmente suplementados com GalOS e/ou fruto-oligossacarídeos, a fim de imitar os múltiplos benefícios dos oligossacarídeos do leite humano. Logo, em vez de adicionar GalOS à fórmula infantil, a outra alternativa (empregada pelos autores) é a de formar tais oligossacarídeos *in situ* durante o tratamento típico do leite com β-galactosidases para eliminar a lactose. Raza *et al.* (2018) também empregaram essa técnica para produzir um iogurte prebiótico com baixo teor de lactose. Primeiramente foi feito a transgalactosilação enzimática do leite, e na sequência, o iogurte. A lactose no iogurte prebiótico (1,44 ± 0,01%) foi estatisticamente menor em comparação com o iogurte controle (3,4 ± 0,02%). Em outras palavras, aproximadamente 60% da lactose foi bioconvertida, resultando nas quantidades de 0,9 g de GalOS, 0,9 g de glicose e 0,55 g de galactose por 100 g de produto. A avaliação organoléptica quanto à cor, consistência e aceitabilidade geral foi estatisticamente similar para ambos os iogurtes, enquanto o sabor e a sinerese foram melhorados no iogurte prebiótico devido aos monossacarídeos e GalOS recém-produzidos, respectivamente. Os autores concluíram que a transgalactosilação da lactose do leite melhora a aceitabilidade do iogurte e é importante para os indivíduos intolerantes à lactose.

A inulina é uma fibra dietética que também se classifica como prebiótica. Frutanos desse tipo são utilizados como ingredientes no desenvolvimento de novos produtos não apenas por suas propriedades nutricionais, mas também devido a razões tecnológicas. A inulina pode ser utilizada como substituto de gordura em alimentos, pois possui a capacidade de promover na boca uma sensação semelhante à da gordura. Seu maior peso molecular quando comparado ao das oligofrutoses por exemplo, torna-a menos solúvel e com habilidade de formar microcristais quando misturada à água ou leite. Esses microcristais não são percebidos na boca, mas interagem para formar uma textura finamente cremosa (PIMENTEL; GARCIA; PRUDENCIO, 2012). Por isso, Nicoletti, Verdi e Endres (2016) utilizaram a inulina para desenvolver um queijo cottage sem lactose com reduzido teor de sódio e gordura (redução de 41 e 30%, respectivamente). Conseguiu-se uma concentração final de inulina no queijo de aproximadamente 5,31% sem alteração das características sensoriais.

Apesar dos seis grupos que compõem o quadro de ingredientes funcionais no Brasil, outras matérias-primas são exploradas a fim de descobrir novos compostos que possam vir a

ter essa alegação. Pesquisadores romenos (DABIJA; ROPCIUC, 2016) estudaram leites fermentados sem lactose adicionados de cranberry e mirtilo. Cranberries são uma importante fonte de vitaminas E, K, C e fibras, além de ter uma boa quantidade de proantocianidinas (COMBS; MCCLUNG, 2017; NEMZER *et al*., 2018), que por sua vez possui efeitos anticancerígenos, bloqueando as bactérias e prevenindo infecções (CÔTÉ *et al*., 2011; YU *et al*., 2016; ALSHAIBANI; ZHANG; WU, 2017). Mirtilos contêm antocianidinas (NEMZER *et al*., 2018; ZHOU *et al*., 2018) que ajudam na reparação do DNA e estimulam a secreção de insulina, razão pela qual são recomendados aos diabéticos (NORBERTO *et al*., 2013; SHI *et al*., 2017). Assim, os autores alegam que o produto obtido é um alimento orgânico funcional que não usa conservantes, aditivos, ou organismos geneticamente modificados.

Rahimi *et al*. (2017) produziram uma bebida láctea sem lactose de toranja, enriquecida com pectina. O produto foi feito a partir de leite permeado obtido da ultrafiltração seguido da hidrólise enzimática. O leite permeado fornece ingredientes nutracêuticos altamente valiosos, incluindo lactoferrina, lactoperoxidase, imunoglobulina, fatores de crescimento, aminoácidos (cadeia essencial e ramificada), lactose, proteínas solúveis, vitaminas e minerais (BRANGER *et al*., 1999). Já o suco concentrado de toranja contém altos níveis de nutrientes, como ácidos fenólicos, licopeno, pectina, minerais e vitaminas; conta também com a presença de antioxidantes naturais, como limonina e naringina (DREWNOWSKI; HENDERSON; SHORE, 1997). Por tudo isso, a bebida desenvolvida é candidata à alegação de propriedade funcional.

Excetuando-se as pesquisas de possíveis ou reais propriedades funcionais dos produtos sem lactose, outros relatos inovadores de derivados lácteos sem lactose foram encontrados. Tonguc e Karagozlu (2017) desenvolveram uma bebida láctea fermentada visando atender consumidores intolerantes à lactose e galactose. Para tal, foi utilizada como base da bebida, uma mistura na proporção de 1:1 de leite sem lactose e dois tipos de fórmula infantil. Os resultados indicaram que o teor de galactose das bebidas foi reduzido a um nível que é adequado para as dietas de pacientes com galactosemia, além do fato das propriedades químicas, microbiológicas e sensoriais destes produtos corresponderem às características comuns de qualidade de um produto lácteo fermentado comercial. Skryplonek *et al*. (2017) elaboraram um *frozen* sem lactose e investigaram os impactos da hidrólise da lactose no produto final. O trabalho foi desenvolvido de modo que a hidrólise enzimática (Ha-lactase) ocorreu simultaneamente com a fermentação. Após a aeração e congelamento, o produto resultante mostrou propriedades de textura e viscosidade otimizadas se comparado com o controle, além de aumento da sua qualidade sensorial. Moynihan *et al*. (2016) combinaram a técnica de ultrafiltração com outras estratégias visando a produção de queijos muçarelas com diferentes e

reduzidos teores de lactose, mas que ao mesmo tempo mantinha constante os níveis de caseína. Por fim, Li *et al*. (2015) estudaram a hidrólise enzimática da lactose em leite integral e desnatado objetivando a produção de leites achocolatados com reduzido teor de açúcar, já que o poder de doçura dos monossacarídeos formados é maior do que o da lactose.

2 INOVAÇÕES NO DESENVOLVIMENTO DE DERIVADOS LÁCTEOS PROBIÓTICOS, PREBIÓTICOS E SIMBIÓTICOS

No Brasil, os alimentos com alegações de propriedades funcionais e ou de saúde são regulamentados pela Agência Nacional de Vigilância Sanitária. Os ingredientes fontes dos nutrientes ou não nutrientes relacionados à alegação de propriedade funcional ou de saúde devem ser comprovadamente seguros para o consumo humano. Ao todo, seis grandes grupos compõem a lista de nutrientes e não nutrientes que tem alegações padronizadas. Fazem parte dessa lista os microrganismos probióticos e alguns compostos alimentares com efeito prebiótico (ANVISA, 2002).

2.1 PROBIÓTICOS

As bactérias probióticas têm atraído a atenção dos biotecnólogos há muito tempo, pois são potencialmente significativas para a saúde, prevenção e tratamento de muitas doenças (KHAMAGAEVA *et al*., 2016). A definição do termo probiótico vem passando por alterações ao longo dos anos devido às constantes pesquisas sobre estes microrganismos e seus efeitos no hospedeiro. O primeiro relato na literatura data de 1965, na ocasião, Lilly e Stillwell (1965) definiram probióticos como compostos, produzidos por um protozoário, que estimulavam o crescimento de outras espécies. Em 2001, a FAO/WHO propôs uma definição que ainda é a mais aceita internacionalmente, todavia, a designação mais recente do termo provém de uma declaração de consenso na qual um painel de especialistas, convocado em 2013 pela Associação Científica Internacional de Probióticos e Prebióticos, discute sobre o uso adequado e o alcance do termo probiótico. Segundo eles, probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios para a saúde ao hospedeiro (HILL *et al*., 2014).

Dentre esses efeitos benéficos podemos destacar três principais, estes podem ser atribuídos a todos os probióticos de forma geral. Nesse contexto, fazemos referência à cepas de várias espécies microbianas bem estudadas e seguras, consumidas pela população através de

alimentos ou suplementos. O efeito principal e também a única alegação dos alimentos probióticos segundo a legislação brasileira é a contribuição para o equilíbrio da microbiota intestinal (ANVISA, 2002). A ANVISA preconiza ainda que a quantidade mínima viável para os probióticos deve estar situada na faixa de 10^8 a 10^9 Unidades Formadoras de Colônias (UFC) na recomendação diária do produto pronto para o consumo, conforme indicação do fabricante.

Outros dois benefícios comuns aos probióticos segundo Hill *et al*. (2014) é servir de suporte a um sistema digestivo saudável e a um sistema imunológico saudável. Além desses efeitos gerais, benefícios específicos de espécies estão sendo descobertos e estudados, o que inclui também diferentes mecanismos de ação que os microrganismos utilizam para desempenhar seu efeito probiótico. O Quadro 3 mostra pesquisas recentes e inovadoras sobre a aplicação dos probióticos, os principais mecanismos envolvidos e os efeitos clínicos observados.

No que diz respeito à *Bifidobacterium animalis* subsp. *lactis* BB-12®, Jungersen *et al*. (2014) fizeram uma extensa revisão a respeito dos mecanismos de ação dessa cepa. Dentre eles, destaca-se a inibição de patógenos, o aumento da função de barreira e as interações imunológicas. Além disso, esta bifidobactéria exibe excelente tolerância ao ácido gástrico e à bile, apresenta naturalmente a hidrolase do sal biliar (uma enzima importante para lidar com as altas concentrações de sal biliar no intestino delgado), e possui fortes propriedades de aderência ao muco. Essas características probióticas são valiosas. BB-12® provou seu efeito benéfico à saúde em vários estudos clínicos sobre saúde gastrointestinal e função imunológica. Estes estudos demonstraram a sobrevivência da BB-12® através do trato gastrointestinal, além de servir como suporte para uma microbiota gastrointestinal saudável. Nesse sentido, este probiótico foi eficaz na melhora do funcionamento intestinal, no efeito protetor contra diarreia, e na redução dos efeitos colaterais do tratamento com antibióticos. Em termos de função imunológica, estudos clínicos demonstraram que a BB-12® aumenta a resistência do corpo a infecções respiratórias comuns, bem como reduz a incidência de infecções agudas do trato respiratório.

Quadro 3 – Algumas linhagens probióticas ou potencialmente probióticas estudadas in vitro, em modelo animal ou em humanos.

Linhagem	**Origem**	**Principais mecanismos de ação envolvidos**	**Benefícios**	**Referências**
Enterococcus faecium FC-K	Kimchi (alimento coreano tradicional fermentado)	Aumento da imunidade (aumento da atividade de macrófagos peritoneais)	Tratamento de alergia (testado em modelo animal).	RHO *et al*., 2017
Enterococcus faecium LZ86	Fezes crianças	Competição pelo sítio de adesão e alteração do metabolismo.	Coloniza as células epiteliais intestinais. Produz vitamina B12 (testes *in vitro*).	LI *et al*., 2017
Enterococcus faecium LCW 44	Leite de camelo cru	Competição pelo sítio de adesão e atividade antimicrobiana		VIMONT *et al*., 2017
Enterococcus faecium CRL 183 e *Bifidobacterium longum* ATCC 15707 (associados)	Cultura iniciadora de queijo e intestino, respectivamente	Produção de compostos antimutagênicos no cólon	Redução dos sintomas de colite (menor grau de inflamação e ulceração no cólon) em modelo animal.	CELIBERTO *et al*., 2017
Entercoccus faecium NCDC124 e *Lactobacillus casei* NCDC299 (isolados ou associados)	Leite em pó e leite, respectivamente	Atividade antimicrobiana	Pode prevenir infecções parasitárias de amebíase e reduz em até 80% a taxa de sobrevivência de *Entamoeba histolytica* (testes *in vitro*).	SARJAPURAM *et al*., 2017

Linhagem	Origem	Principais mecanismos de ação envolvidos	Benefícios	Referências
Lacticaseibacillus rhamnosus		Atividade antimicrobiana e aumento da imunidade	Diminuição da infecção por *Candida albicans* (testes *in vivo* com larvas de *Galleria mellonella*).	DE OLIVEIRA *et al*., 2017
Lactobacillus casei Shirota		Atividade antimicrobiana	Prevenção de cáries dentárias (teste em crianças).	LIN *et al*., 2017
Lacticaseibacillus paracasei		Atividade antimicrobiana	Prevenção de cáries dentárias (teste em crianças).	LIN *et al*., 2017
Lacticaseibacillus paracasei	Produtos lácteos caseiros tradicionais da Mongólia Interior, China	Aumento da imunidade	Reduz o risco de infecções agudas do trato respiratório superior no idoso e pessoas de meia-idade.	PU *et al*., 2017
Bifidobacterium bifidum G9-1	Fezes crianças	Alteração do metabolismo	Prevenção da mucosite intestinal durante o tratamento quimioterápico de câncer (testado em modelo animal).	KATO *et al*., 2017
Bifidobacterium animalis subsp *lactis* LKM512	TGI Animal	Neutralização de compostos indesejáveis (redução dos níveis de trimetilamina).	Reduz o risco de desenvolvimento da arteriosclerose (teste em voluntários).	MATSUMOTO *et al*., 2017

Fonte: Os autores

2.1.1 Derivados lácteos probióticos

Nos últimos anos, mais e mais bactérias probióticas foram incorporadas nos alimentos como adjuvantes dietéticos (SHAH, 2011). O Quadro 4 descreve aplicações de bactérias probióticas em ampla gama de derivados lácteos com diferentes objetivos de estudo. Do ponto de vista funcional, é essencial que os microrganismos probióticos estejam presentes em quantidades suficientes no momento do consumo, permitam a sua produção em larga escala, sobrevivam durante o processamento industrial e também durante o armazenamento do produto em que são adicionados (TRIPATHI; GIRI, 2014). Além disso, os produtos lácteos são considerados uma excelente matriz para transportar bactérias probióticas para o trato gastrointestinal humano, uma vez que a gordura, as proteínas, os açúcares e o pH desses produtos favorecem a manutenção e a sobrevivência dos microrganismos (RANADHEERA; BAINES; ADAMS, 2010). No entanto, um dos principais desafios no desenvolvimento de um produto probiótico é assegurar uma alta taxa de sobrevivência das bactérias durante a fabricação do produto e durante a vida de prateleira, além de poder sobreviver durante a passagem pelo trato gastrointestinal humano (GRANATO *et al*., 2010; TRIPATHI; GIRI, 2014; ZARE *et al*., 2012).

Quadro 4 – Alguns exemplos de incorporação de bactérias probióticas em derivados lácteos.

Produto	**Cepa probiótica**	**Matéria-Prima**	**Referência**
Queijo Minas Frescal	*Bifidobacterium* BB-12	Leite de búfala	(VERRUCK *et al*., 2015a)
Queijo Minas Frescal	*Bifidobacterium* BB-12	Leite de búfala	(VERRUCK *et al*., 2015b)
Queijo Minas Frescal	*Bifidobacterium* BB-12	Leite de vaca	(FRITZEN-FREIRE *et al*., 2010)
Queijo *Scamorza*	*Bifidobacterium longum*, *Bifidobacterium lactis*, e *Lactobacillus acidophilus*	Leite de ovelha	(ALBENZIO *et al*., 2013a)
Queijo *Scamorza*	*Bifidobacterium longum* 46, *Bifidobacterium lactis* BB-12, e *Lactobacillus acidophilus* LA-5	Leite de ovelha	(ALBENZIO *et al*., 2013b)
Queijo *Pecorino*	*Bifidobacterium longum* 46, *Bifidobacterium lactis* BB-12, e *Lactobacillus acidophilus* LA-5	Leite de ovelha	(ALBENZIO *et al*., 2010)
Queijo *Pecorino*	*Bifidobacterium longum* 46, *Bifidobacterium lactis* BB-12, e *Lactobacillus acidophilus* LA-5	Leite de ovelha	(SANTILLO; ALBENZIO, 2008)
Queijo *Pecorino*	*Bifidobacterium longum* 46, *Bifidobacterium lactis* BB-12, e *Lactobacillus acidophilus* LA-5	Leite de ovelha	(SANTILLO; ALBENZIO, 2015)
Queijo coalho	*Lactobacillus acidophilus, Lactobacillus paracasei* e *Bifidobacterium* BB-12	Leite de cabra	(BEZERRA *et al*., 2017)

Produto	Cepa probiótica	Matéria-Prima	Referência
Queijo coalho	*Lactobacillus acidophilus* LA-5	Leite de cabra	(DOS SANTOS *et al*., 2012)
Queijo *Canestrato Pugliese*	*Bifidobacterium bifidum* Bb02 e *Bifidobacterium longum* Bb46	Leite de ovelha	(CORBO *et al*., 2001)
Queijo Prato	*Lactobacillus casei* 01	Leite de vaca	(SILVA *et al*., 2017)
Queijo Argentino	*Lactobacillus acidophilus* La-5 e *Bifidobacterium animalis* ssp. *lactis* Bb12	Leite de ovelha	(PEROTTI *et al*., 2014)
Queijo cremoso	*Lactobacillus acidophilus* La-5 e *Bifidobacterium animalis* ssp. *lactis* Bb12	Leite de ovelha	(CUFFIA; BERGAMINI; CANDIOTI, 2018)
Queijo ricota	*Lactobacillus acidophilus* La-5 e *Bifidobacterium animalis* ssp. *lactis* Bb12	Leite de cabra e soro de leite de cabra	(MEIRA *et al*., 2015)
Iogurte	*Lactobacillus acidophilus* LA-5*, Bifidobacterium animalis* subsp. *lactis* BB-12 e *Propionibacterium jensenii* 702	Leite de cabra	(SENAKA RANADHEERA *et al*., 2012)
Iogurte	*Lactobacillus casei* ATCC393 e *Lactobacillus bulgaricus* DSM20081	Leite de vaca	(TERPOU *et al*., 2017)
Iogurte	*Lactobacillus acidophilus*	Leite de cabra	(MACHADO *et al*., 2017)

Produto	Cepa probiótica	Matéria-Prima	Referência
Iogurte	*L. casei* ATCC 393	Leite de vaca	(BOSNEA *et al*., 2017)
Iogurte	*Lactobacillus paracasei* FNU	Leite de vaca	(ILHA *et al*., 2016)
Iogurte	*Lactobacillus paracasei* ATCC 10746	Leite de vaca	(SCARIOT *et al*., 2018)
Iogurte	*Lactobacillus casei* PRA205 e *Lactobacillus rhamnosus* PRA331	Leite de vaca	(RUTELLA; TAGLIAZUCCHI; SOLIERI, 2016)
Iogurte Grego	*Lactobacillus paracasei* subsp. *paracasei* DC412	Leite de ovelha	(PAPADIMITRIOU *et al*., 2007)
Iogurte/ Leite fermentado	*Lactobacillus acidophilus*, *Lactobacillus casei* e *Lactobacillus reuteri*	Leite de vaca	(MANI-LÓPEZ; PALOU; LÓPEZ-MALO, 2014)
Leite fermentado	*Lactobacillus plantarum* 69	Leite de cabra	(CHEN *et al*., 2018)
Leite fermentado	*Lactobacillus acidophilus* La-5 e *Bifidobacterium animalis* ssp. *lactis* Bb12	Leite de camelo fêmea, de vaca, de cabra e de ovelha	(VARGA; SÜLE; NAGY, 2014)
Leite fermentado	*Lactobacillus acidophilus* La-5 e *Bifidobacterium animalis* ssp. *lactis* Bb12	Leite de vaca e Leite de cabra	(COSTA *et al*., 2015)

Produto	Cepa probiótica	Matéria-Prima	Referência
Bebida láctea fermentada	*Bifidobacterium animalis* subsp. *lactis* e *Lactobacillus rhamnosus*	Leite de cabra e soro de leite de cabra	(BURITI *et al*., 2014)
Kefir Torula®	*Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus* biovar. *diacetylactis*, *Leuconostoc mesenteroides* ssp. cremoris, *Lactobacillus plantarum*, *Lactobacillus casei*, e a levedura *Kluyveromyces marxianus* var. *fragilis*	Leite de cabra	(QUIRÓS *et al*., 2005)
Kefir Tibetano	*Lactobacillus acidophilus* LA15, *Lactobacillus plantarum* B23 e *Lactobacillus kefiri* D17	Leite de vaca	(ZHENG *et al*., 2013)
Kefir	*Lactobacillus kefiri*	Leite de vaca	(CARASI *et al*., 2014)
Frozen Iogurte	*Bifidobacterium animalis* subsp. *lactis* BI-07	Leite de cabra	(BEZERRA *et al*., 2015)
Sorvete	*Bifidobacterium animalis* subsp. *lactis* BI-07	Leite de cabra	(SILVA *et al*., 2015)

Fonte: Os autores

Durante o período de processamento até o consumo de um alimento, os probióticos precisam ser protegidos contra condições de processamento, como alta temperatura e cisalhamento; dessecação, se aplicada a um alimento desidratado; condições de armazenamento (temperatura, umidade, oxigênio) e; degradação no trato gastrointestinal, especialmente por causa do baixo pH dos ácidos estomacais e sais biliares no intestino delgado (MANOJLOVIĆ *et al*., 2010).

A fim de exercer os efeitos benéficos sobre o hospedeiro, além de estar em contagem suficiente no produto, é necessário que os microrganismos probióticos possam superar a passagem através do sistema digestivo humano (SHAH, 2011). Uma vez que os efeitos dos probióticos estão diretamente relacionados à sua atividade no trato digestivo, e estes dependem de sua colonização e sobrevivência neste ambiente, essas bactérias devem ser resistentes aos processos fisiológicos e físico-químicos do sistema gastrointestinal. Portanto, as bactérias probióticas devem sobreviver à passagem através da boca, esôfago, estômago (pH 2) e intestino delgado para exercer seus benefícios no intestino. Assim, elas devem sobreviver ao suco gástrico (ácido clorídrico), suco pancreático e sais biliares (ORTAKCI *et al*., 2012).

Durante o trânsito através das diferentes seções do trato gastrointestinal, os probióticos estão expostos a diferentes condições de estresse (YEO *et al*., 2011). As perdas ocorrem ao longo de todo o trato gastrointestinal, mas é evidente que o ambiente ácido do estômago e a presença de bile no duodeno são fatores importantes que afetam a viabilidade das bactérias probióticas (MAINVILLE; ARCAND; FARNWORTH, 2005). No estômago, muitas estirpes de *Lactobacillus* spp. e *Bifidobacterium* spp. intrinsecamente perdem a capacidade de sobreviver a essa acidez. Em geral, a tolerância ácida das bifidobactérias pode ser considerada fraca, com exceção de *Bifidobacterium lactis* e *Bifidobacterium animalis* (MATSUMOTO; OHISHI; BENNO, 2004). A sobrevivência de bactérias que não possuem uma via respiratória, como *Bifidobacterium* BB-12, está associada à capacidade da enzima F0F1-ATPase de manter o pH intracelular em condições ácidas (SÁNCHEZ *et al*., 2007). Devido aos efeitos prejudiciais, os probióticos, mesmo que ainda sejam viáveis no estômago, podem estar injuriadas ao atingir o cólon e suas chances de sobrevivência podem ser comprometidas, além de comprometer sua capacidade de colonizar o intestino e ter qualquer efeito benéfico sobre o hospedeiro (YEO *et al*., 2011). Esse comportamento pode ser atribuído ao baixo valor de pH (~ 2) e à presença de pepsina no estômago (VERRUCK *et al*., 2015b). Além disso, a tolerância ácida da bactéria probiótica depende de fatores como meio de crescimento, condições de incubação, perfil de enzimas e composição da membrana citoplasmática de cada estirpe (MATSUMOTO; OHISHI; BENNO, 2004).

Após a exposição às condições do estômago, as bactérias probióticas atingem o intestino delgado e são submetidas aos sais biliares, pancreatina e pH aproximadamente 5,0 (GUERRA *et al*., 2012). Os sais biliares são conhecidos por sua atividade antimicrobiana contra bactérias probióticas, principalmente devido à sua natureza anfifílica e capacidade de dissolver a membrana celular bacteriana (MADUREIRA *et al*., 2011). Além disso, os sais biliares se acumulam no citoplasma bacteriano, causando distúrbios na integridade da membrana e, portanto, a morte celular (KURDI *et al*., 2006). A sobrevivência do probiótico no ambiente biliar depende da concentração de bile, tempo de exposição e estirpes bacterianas (VINDEROLA; REINHEIMER, 2003). Além disso, a sobrevivência de estirpes probióticas no trato gastrointestinal pode não só depender de seu número e estado fisiológico, mas também da matriz alimentar e do consumo de alimentos que afetam a excreção biliar (SIRÓ, 2011). Aspectos como quantidade e tipo de proteína e gordura, pH, presença de certos carboidratos ou outros ingredientes também podem influenciar a resistência de probióticos durante a passagem pelo trato gastrointestinal (BEDANI *et al*., 2013; BURNS *et al*., 2014; CASAROTTI; TODOROV; PENNA, 2015; SENDRA *et al*., 2016).

Após a passagem através do estômago e do duodeno, outro aspecto necessário em relação às bactérias probióticas é a sua capacidade de aderir à superfície epitelial e colonizar o intestino delgado ou o cólon (CANZI *et al*., 2005). A superfície bacteriana hidrofóbica é essencial para a interação entre a camada intestinal da glicoproteína, receptor da célula epitelial intestinal e os locais de ligação de ácidos graxos (DIANAWATI; MISHRA; SHAH, 2015). Além disso, a presença de grupos apolar predominantes de membrana de bifidobactérias também pode suportar a aderência. A hidrofobicidade superficial de *B. animalis* BB-12, *L. acidophilus* NCFM e *L. rhamnosus* GG foram de 50, 8 e 20%, respectivamente (WANG *et al*., 2010). No entanto, a capacidade de adesão varia com a cepa utilizada e os danos anteriores sofridos (CANZI *et al*., 2005). A eficácia da cultura probiótica pode ser influenciada pela matriz do alimento/suplemento no qual ela é inserida (LEE *et al*., 2015). Ratos que tiveram colite ulcerativa induzida e receberam células de *Lactobacillus casei* BL23 suspendidas em leite ao invés de suspendidas em tampão fosfato-salino (PBS) apresentaram melhores resultados contra o desenvolvimento da doença. Assim, a atenuação da colite foi considerada dependente da matriz láctea pela qual a cepa é carreada ao intestino. Esses resultados indicam fortemente que os produtos lácteos podem ser a matriz de entrega preferencial para cepas probióticas para beneficiar a saúde humana.

Sendo assim, a aplicação em derivados lácteos com a finalidade de proteger a bactéria probiótica ao longo do processamento, armazenamento e consumo do produto já é uma

realidade bem-sucedida. A incorporação de *Bifidobacterium* BB-12 em iogurte grego não afetou as características gerais do produto, e os dados microbiológicos sugeriram que o iogurte grego é uma boa matriz para manter a viabilidade de *Bifidobacterium* BB-12 durante o armazenamento (PINTO *et al*., 2017). Além disso, a produção de queijo itálico probiótico foi levada a nível industrial, descrevendo completamente um sistema atrativo para exploração comercial (BLAIOTTA *et al*., 2017). Ademais, a literatura relata outros meios e tecnologias (ver tópico 3 deste capítulo) que visam a proteção do probiótico durante o processamento, armazenamento e passagem do produto através do trato gastrointestinal.

A interação entre probióticos e outros microrganismos em produtos lácteos, no intuito de melhorar a viabilidade dos probióticos, também vem sendo estudada (YEO; TOH; LIU, 2016). Foi examinada a viabilidade de três cepas diferentes de *Bifidobacterium lactis* quando inoculadas em leite UHT com e sem a presença da levedura *Williopsis saturnus* var. *saturnus* NCYC 22. Na presença da levedura co-inoculada, a contagem de células viáveis de *B. lactis* para cepas HN019 e BB-12 manteve-se acima de 6 log UFC/mL, enquanto a estirpe B94 apresentou 5,7 log UFC/mL. Ao se co-incubar levedura e *B. lactis* HN019 no leite sob condição anaeróbia, constatou-se que não houve melhora da sobrevivência, indicando que a remoção de oxigênio pode não ser responsável pela propriedade de reforço de viabilidade de *W. saturnus* NCYC 22. A adição de sobrenadante de levedura ou levedura não viável também não mostrou efeitos estabilizadores, sugerindo que o contato físico e/ou a interação entre *W. saturnus* viáveis e *B. lactis* desempenham um papel importante na manutenção da viabilidade do probiótico. *W. saturnus* NCYC 22 poderia aumentar a sobrevivência de *B. lactis* no leite à temperatura ambiente, independentemente da concentração inicial de células de levedura inoculadas, devido ao crescimento da mesma. Esta interação poderia ajudar a prolongar a vida útil de bebidas lácteas contendo bifidobactérias probióticas.

2.2 PREBIÓTICOS

Apesar dos alimentos prebióticos serem estudados e consumidos há muito tempo, a utilização do termo prebiótico é recente, e sua definição foi discutida e refinada várias vezes desde que foi introduzida pela primeira vez em 1995 por Gibson e Roberfroid (1995). Uma designação que foi amplamente difundida pela comunidade científica internacional relata que o prebiótico é um ingrediente seletivamente fermentado que resulta em mudanças específicas na composição e/ou atividade da microbiota gastrointestinal, conferindo benefício(s) sobre a saúde do hospedeiro (GIBSON *et al*., 2010). Este conceito foi revisto pela Associação

Científica Internacional para Probióticos e Prebióticos (*International Scientific Association for Probiotics and Prebiotics) e a definição mais recente de prebiótico o considera u*m substrato utilizado seletivamente por microrganismos hospedeiros que conferem benefício para a saúde (GIBSON *et al*., 2017). Aparentemente esta nova definição não difere muito da anterior, porém, os autores esclarecem que:

- ✓ Embora a maioria dos prebióticos atuais sejam administrados por via oral, eles também podem ser administrados diretamente a outros locais do organismo colonizados por microrganismos, como o trato vaginal e a pele;
- ✓ Os efeitos de saúde dos prebióticos estão evoluindo, mas atualmente incluem benefícios para o trato gastrointestinal (por exemplo, inibição de agentes patogênicos, estimulação imune), cardiometabolismo (por exemplo, redução dos níveis de lipídios no sangue, efeitos sobre a resistência à insulina), saúde mental (por exemplo, metabólitos que influenciam a função cerebral, energia e cognição) e ossos (por exemplo, biodisponibilidade mineral), dentre outros;
- ✓ Os prebióticos atualmente mais conhecidos são carboidratos, mas outras substâncias, como polifenóis e ácidos graxos poliinsaturados convertidos em seus respectivos ácidos graxos conjugados, podem entrar na definição atualizada, quando assumido peso convincente da evidência no hospedeiro alvo;
- ✓ O(s) efeito(s) benéfico(s) de um prebiótico na saúde deve ser confirmado para o uso pretendido *in vivo* e mediado através da microbiota.

Portanto, a classe mais importante dos prebióticos representa um tipo especial de fibra dietética que, quando fermentada, provoca alterações mensuráveis da composição da microbiota intestinal, geralmente um aumento do número de bactérias consideradas benéficas, como bifidobactérias ou certos produtores de butirato (MARCHESI *et al*., 2016). Entretanto, desde a introdução do conceito, muitos ingredientes alimentícios, notoriamente oligossacarídeos e polissacarídeos – incluindo fibra vegetal – tem sido clamados como tendo atividade prebiótica sem se considerar algumas características importantes. Nem todos os carboidratos da dieta podem ser considerados prebióticos, logo, é necessário definir alguns critérios para designar um ingrediente como tal:

- ✓ Não ser hidrolisado nem absorvido na parte superior do trato gastrointestinal;
- ✓ Ser fermentado pela microbiota intestinal;
- ✓ Apresentar estimulação seletiva ao crescimento e/ou atividade de bactérias intestinais contribuindo para a saúde do hospedeiro.

Um dos principais mecanismos pelo qual os prebióticos são considerados promotores de benefícios para a saúde é a sua influência na produção de ácidos graxos de cadeia curta, que por sua vez possuem atividade antimicrobiana e reduzem o pH intestinal (e, portanto, excluem agentes patogênicos), e ainda acarreta em vários efeitos benéficos fisiológicos, metabólicos e imunológicos (BINDELS *et al*., 2015). Há na literatura, resultados convincentes e reprodutíveis de estudos em animais que demonstram eficácia na prevenção ou tratamento de muitas doenças (por exemplo, síndrome do intestino irritável, câncer do cólon, obesidade, diabetes tipo 2 e doenças cardiovasculares) (MARCHESI *et al*., 2016). Um estudo clínico investigou a administração de prebiótico NutraFlora® em pacientes com lesão cerebral traumática. Este estudo sugeriu que os pacientes que receberam fórmulas com maior quantidade de prebiótico são mais propensos a ter maiores níveis de pré-albumina, o que pode refletir numa melhor nutrição ao longo de sua internação hospitalar, podendo obter também algum benefício em relação às taxas de infecções, particularmente a bacteremia (PAINTER; RICKERDS; ALBAN, 2015). Todavia, estabelece-se a crítica de que os testes em seres humanos são insuficientes e suspeitos, isso porque no campo dos prebióticos há menos estudos clínicos bem desenvolvidos ou bem projetados em comparação com probióticos.

2.2.1 Derivados lácteos prebióticos

A utilização de prebióticos pode resultar em alterações dos atributos de qualidade de produtos lácteos devido a interações entre o ingrediente funcional e os componentes da matriz alimentar. Os principais prebióticos utilizados para aplicação em derivados lácteos são a inulina e a oligofrutose. As diferentes variações de DP nos produtos industriais estão diretamente ligadas às propriedades tecnológicas da inulina e oligofrutose quando aplicadas em diferentes produtos (MENSINK *et al*., 2015). Frutanos tipo inulina são utilizados como ingredientes no desenvolvimento de novos produtos não apenas por suas propriedades nutricionais, mas também devido a razões tecnológicas. A inulina tem um sabor ligeiramente doce (menos de 10% de sacarose), dependendo do comprimento da cadeia, e apresenta solubilidade moderada em água (10% à temperatura ambiente) (KARIMI *et al*., 2015). A inulina pode ser utilizada como substituto de gordura em derivados lácteos, pois possui a capacidade de promover na boca uma sensação semelhante à da gordura. Seu maior peso molecular quando comparado ao das oligofrutoses torna-a menos solúvel e com habilidade de formar microcristais quando misturada à água ou leite. Esses microcristais não são percebidos na boca, mas interagem para formar uma textura finamente cremosa (PIMENTEL; GARCIA; PRUDENCIO, 2012).

Oligofrutoses têm propriedades comparáveis às do açúcar e xaropes de glicose, por possuírem maior quantidade de açúcares livres. A doçura na forma pura é de 30 a 35% quando comparada à sacarose e apresenta baixo valor calórico (1-2 kcal/g) (PIMENTEL; GARCIA; PRUDENCIO, 2012). Assim, podem ser aplicados na substituição de carboidratos para gerar produtos lácteos com teor reduzido de açúcar. Por estas razões, a inulina e a oligofructose são amplamente aplicadas na indústria de laticínios (KARIMI *et al*., 2015). Em iogurtes e leites fermentados a aplicação de prebióticos tem sido descrita demonstrando resultados promissores. O Quadro 5 descreve aplicações de agentes prebióticos em leites fermentados e bebidas lácteas e os resultados encontrados.

Quadro 5 – Alguns exemplos de aplicação de agentes prebióticos em leites fermentados e bebidas lácteas.

Produto	**Prebiótico**	**Resultado**	**Referência**
Leite fermentado	Inulina	Produto com maior tendência para uma coloração esverdeada. Menor índice de sinerese e maior firmeza.	(DEBON *et al*., 2012)
Bebida láctea de graviola	Inulina	Amostras contendo inulina com maior grau de polimerização (DP ≥ 23) apresentaram melhor estabilidade física do que as demais (DP ≥ 10).	(GUIMARÃES *et al*., 2018)
Iogurte de leite ovelha	Inulina	Observou-se menores valores de pós-acidificação. O iogurte constituiu-se numa grande fonte de ácido oleico e isômero ácido linoleico conjugado cis-9,trans-11.	(BALTHAZAR *et al*., 2016)
Iogurte com baixo teor de gordura	Inulina e frutanos de agave	Melhorou os atributos sensoriais do iogurte. A inulina formou estruturas geleificadas enquanto os frutanos cobriram as micelas de caseína.	(CRISPÍN-ISIDRO *et al*., 2015)
Iogurte com baixo teor de gordura	Inulinas com diferentes graus de polimerização.	Melhora da sensação cremosa na boca.	(KIP *et al*., 2006)
Iogurte com baixo teor de gordura	Inulina	Aumentou a separação do soro e consistência. A tirosina e os níveis de ácidos graxos voláteis foram afetados negativamente.	(GUVEN, KARACA, HAYALOGLU; 2005)
Iogurte desnatado	Inulina	Diminuiu sinérese e melhorou características de corpo e textura.	(ARYANA *et al*., 2007)

Produto	Prebiótico	Resultado	Referência
Iogurte desnatado	Inulinas com diferentes graus de polimerização.	Diminuição dos valores de firmeza, viscosidade aparente e tensão de escoamento. Inulina de cadeia longa demonstrou um comportamento reológico mais próximo do iogurte controle (integral).	(SHERKAT, 2008)
Iogurte	Oligofrutose	Não houve influência sobre o pH, a proteólise ou a viabilidade de *Streptococcus thermophilus e Lactobacillus bulgaricus* durante 28 dias de armazenamento refrigerado.	(CRUZ *et al*., 2013)
Iogurte	Fibra de espargos	Aumentou a consistência. Fibras diminuíram a claridade e atribuíram uma coloração amarelo-esverdeada.	(SANZ *et al*., 2008)
Iogurte	Inulina e fibras comerciais de maçã, trigo e bambu (testadas como potenciais prebióticos).	Não houve diferença na sinérese e no pH. Boa aceitabilidade por parte dos analistas do painel.	(STAFFOLO *et al*., 2004)

Fonte: Os autores.

Inulina e frutooligossacarídeo provaram ser uma alternativa promissora como substitutos de gordura na formulação de sorvete de leite de ovelha, devido às propriedades reológicas, tais como dureza, viscoelasticidade e consistência similares ao produto com gordura. Essas mesmas formulações prebióticas foram também percebidas mais cremosas e brilhantes do que a amostra controle. Além disso, a maioria dos sorvetes prebióticos foram mencionados como sendo mais doces do que o sorvete com gordura, o que sugere que essas fibras podem atuar como edulcorantes (BALTHAZAR *et al*., 2017). Sendo assim, a substituição da gordura do leite por prebióticos para a fabricação de sorvete pode ser uma alternativa efetiva para melhorar os aspectos nutricionais e fisiológicos, devido ao baixo valor calórico e funcionalidade oferecidos pelos prebióticos (AKBARI *et al*., 2016). Além disso, a inulina também pode ser utilizada como controladora de fenômenos de cristalização e recristalização em produtos lácteos congelados (SOUKOULIS; LEBESI; TZIA, 2009).

A inulina também tem sido amplamente utilizada em queijos como substituto de gordura (KARIMI *et al*., 2015). A remoção de gordura do queijo causa defeitos reológicos, de textura, funcionais e sensoriais, como característica de textura de borracha, falta de sabor, amargor, sabor desagradável, fraca capacidade de fusão e cor indesejável (O'CONNOR; O'BRIEN, 2011). Assim, é um desafio tecnológico utilizar substitutos de gordura que mantenham as mesmas propriedades funcionais e organolépticas que os queijos sem redução no seu teor de gordura (FADAEI *et al*., 2012). Nesse sentido, a inulina parece ser particularmente adequada para a substituição de gordura em queijos de baixo teor de gordura, pois pode contribuir para melhorar a sensação de maciez na boca (MEYER *et al*., 2011). Essa sensação de cremosidade é gerada através da encapsulação de uma grande quantidade de água e complexação com agregados proteicos (BOT *et al*., 2004; KIP; MEYER; JELLEMA, 2006).

A inulina de cadeia longa e alto peso molecular é a mais indicada para ser utilizada como substituto de gordura. Comprimentos de cadeia mais longos reduzem a solubilidade da inulina e resultam na formação de microcristais de inulina quando misturado com o leite. A propriedade da inulina de alto DP de mimetizar o efeito da gordura é o dobro do que a inulina padrão, além desta não apresentar doçura residual (NINESS, 1999). Um queijo Kashar fresco com redução de 70% gordura utilizando inulina de cadeia longa foi produzido. A adição de 5% de inulina resultou em dureza menor em comparação com o queijo controle. Este efeito amaciante poderia ser atribuído tanto à maior proporção de umidade quanto à proteína e ao aumento no volume do queijo, o que diminui a quantidade de proteína matriz (KOCA; METIN, 2004). Além disso, a proporção de 10% de inulina foi suficiente para obter um queijo cremoso de baixo teor de gordura com atributos químicos próximos aos do queijo com alto teor de

gordura que não contém inulina (FADAEI *et al*., 2012). Quando comparadas a outras fibras, a inulina teve melhores resultados sobre a substituição de gordura em queijos muçarela do que metoxipectina, polidextrose e amido resistente (WADHWANI, 2011). O efeito da substituição da gordura por 2, 3 e 7% de inulina de cadeia longa (DP> 23) nas propriedades texturiais e microestruturais de um queijo fresco de leite de cabra também foram avaliadas (SALVATORE *et al*., 2014). As amostras de queijo contendo inulina apresentaram estrutura mais aberta em comparação com queijos controles devido à diminuição da distribuição de gordura na matriz de proteína. O posicionamento da inulina na rede de caseína apareceu incorporada no sistema de gel e cujo tamanho aumentou com a maior concentração de inulina no queijo. A inulina interrompe a rede de caseína, resultando em um efeito amaciador na estrutura do gel (SALVATORE *et al*., 2014).

Os galactooligossacarídeos podem ser facilmente adicionados em derivados lácteos, tais como em sorvetes, iogurtes, leitelho desidratado e bebidas lácteas devido à sua excelente solubilidade. No sorvete os galactooligossacarídeos apresentam efeito positivo sobre as características físico-químicas, óticas e sensoriais. Em geral, há aumento da firmeza e diminuição da taxa de fusão, o que contribui para uma maior estabilidade do produto. Do ponto de vista sensorial, os sorvetes suplementados com galactooligossacarídeos se destacaram pelos atributos de sabor e textura (BALTHAZAR *et al*., 2015). Em iogurte, os galactooligossacarídeos podem ser adicionados antes ou após fermentação, sendo que a estrutura do iogurte torna-se mais lisa e mais cremosa. Além disso, as bactérias usadas para fabricação de iogurte não utilizam os galactooligossacarídeos como fonte de carbono, o que faz com que ele não seja metabolizado até atingir o intestino grosso (SANGWAN *et al*., 2011). Pode-se ainda produzir leitelho em pó contendo galactooligossacarídeos em sua formulação. Este produto pode ser facilmente utilizado, por exemplo, na fabricação de derivados lácteos fermentados (ČURDA *et al*., 2006).

Os xilooligossacarídeos também têm sido utilizados na formulação de derivados lácteos prebióticos. Os aspectos físico-químicos e sensoriais de iogurte enriquecido com xilooligossacarídeos foram comparados àqueles com iogurtes contendo gelatina. O pH, a acidez e os sólidos totais foram significativamente afetados pela adição de xilooligossacarídeos. A adição de até 3,5% de xilooligossacarídeos não influenciou o gosto e a aceitabilidade geral, porém contribuíram com sabor residual (MUMTAZ *et al*., 2008). Além disso, o iogurte enriquecido com xilooligossacarídeos mostrou melhora significativa na absorção de minerais e redução no teor de glicose em ratos albinos (MUMTAZ *et al*., 2009). Sendo assim, os xilooligossacarídeos podem ser incorporados como ingrediente funcional na formulação de

produtos lácteos com benefícios para a saúde. A adição de xilooligossacarídeos na fabricação de requeijão cremoso também foi investigado. A adição de xilooligossacarídeos resultou em uma estrutura mais densa e compacta, com maior viscosidade aparente, elasticidade e firmeza. A adição de xilooligossacarídeos melhorou as propriedades reológicas e físico-químicas (diminuição da viscosidade e tamanho de partícula e aumento da taxa de fusão) e características sensoriais (melhora no sabor salgado e ácido, maior homogeneidade e menor gosto amargo) (FERRÃO *et al*., 2018).

2.3 SIMBIÓTICOS

A associação de um ou mais probióticos com um ou mais prebióticos dá origem a um produto denominado simbiótico. Os prebióticos são complementares e sinérgicos aos probióticos, apresentando assim fator multiplicador sobre suas ações isoladas. Essa combinação deve possibilitar a sobrevivência da bactéria probiótica no alimento e nas condições do meio gástrico, propiciando sua ação no intestino (FLESCH; POZIOMYCK; DAMIN, 2014).

O consumo em associação de probióticos e de prebióticos selecionados apropriadamente pode aumentar os efeitos benéficos de cada um deles. A inulina e os frutooligossacarídeos apresentam efeito bifidogênico, ou seja, estimulam a proliferação das bifidobactérias no intestino, as quais, por efeito antagonista, suprimem a atividade de outras bactérias, como a *Escherichia coli*, *Enterococcus faecalis* e o gênero *Proteus*. A proliferação de bifidobactérias estimulada pelos frutooligossacarídeos leva à redução do pH em virtude da produção de ácidos, tendo como efeito a diminuição do número de bactérias patogênicas ou nocivas, consequentemente atenuando a formação de metabólitos tóxicos (OLIVEIRA, 2009).

A potencial sinergia entre probióticos e prebióticos foi considerada eficiente devido à melhora da sobrevivência e implantação dos probióticos no sistema gastrointestinal. Existem muitos exemplos de simbióticos relatados na literatura científica com dados de observações *in vitro* (por exemplo, linhagem *L. casei* Shirota + oligomate 55 ™; *L. acidophilus* ATCC 4962 + manitol, frutooligossacarídeo e inulina; *Lactobacillus sakei* JCM + frutooligossacarídeo e trealose; *L. plantarum* e *L. acidophilus* + xilooligossacarídeo e frutooligossacarídeo) e *in vivo* (*B. longum* + oligofrutose; *B. breve* cepa Yakult + galactooligossacarídeo; Lafti ™ B94 + amido resistente; e *Lactobacillus gasseri* + inulina e oligossacarídeos não especificados) (ANADÓN *et al*., 2016; FURRIE *et al*., 2005).

Os simbióticos, assim como os antibióticos, têm potencial para controlar infecções, devido à produção de substâncias resultantes da fermentação por probióticos no intestino grosso

(BENGMARK; GARCÍA DE LORENZO; CULEBRAS, 2001). Os simbióticos também são considerados úteis para o aprimoramento da funcionalidade das barreiras epiteliais e para modificação no ecossistema bacteriano (RITCHIE; ROMANUK, 2012). Embora os probióticos possam atuar também no intestino delgado; os prebióticos por sua vez são direcionados exclusivamente para melhorar a microbiota no intestino grosso. Em um produto simbiótico ideal, uma relação sinérgica é desenvolvida entre microrganismos vantajosos (probióticos) e substrato selecionado (prebióticos) e, devido à essa relação sinérgica, a atividade probiótica é aumentada.

2.3.1 Derivados lácteos simbióticos

Na literatura há inúmeros trabalhos envolvendo a produção de derivados lácteos simbióticos, uma vez que os lácteos representam um excelente meio para carrear ou gerar culturas vivas e ativas. No Quadro 6 são descritos exemplos de aplicação de bactérias probióticas em associação com prebióticos em derivados lácteos, bebidas lácteas e iogurte são as matrizes mais exploradas. A utilização dos probióticos e prebióticos em simbiose nos derivados lácteos pode multiplicar os efeitos positivos desse alimento no hospedeiro. Em um estudo randomizado, indivíduos que consumiram iogurte complementado com 0,6% de isomaltooligossacarídeo e duas cepas probióticas, *B. lactis* Bi-07 e *L. acidophilus* NCFM apresentaram aumento das populações de lactobacilos e bifidobactérias no cólon. Além disso, o iogurte simbiótico poderia ser usado como alimento funcional para promover respostas imunes (WANG *et al*., 2012). A capacidade de um leite fermentado simbiótico contendo isomaltooligossacarídeo e *Lactobacillus paracasei* 01 para proteger as células epiteliais *in vitro* foi avaliada. Os autores concluíram que a bebida simbiótica fermentada com *L. paracasei* 01 (10^8 UFC/mL) e isomaltooligossacarídeo (1%) é capaz de fornecer efeitos protetores sobre células intestinais (CHEN *et al*., 2016). Além disso, a suplementação de kefir com isomaltooligossacarídeo (4%) aumentou a taxa de acidificação e diminuiu o tempo de fermentação. Assim, os kefirs suplementados com isomaltooligossacarídeo podem reduzir os custos de produção reduzindo o tempo de fermentação (OH *et al*., 2013).

Devido à uma grande aceitação do consumidor, as sobremesas lácteas de chocolate podem representar novas alternativas para produtos simbióticos (VALENCIA *et al*., 2016). Além disso, durante o desenvolvimento de queijos cremosos simbióticos de leite de cabra a inulina apresentou interferência em alguns parâmetros de textura, como a melhoria da consistência de queijo e sorvete. Fibras dietéticas com elevada capacidade de retenção de água

podem ser usadas para evitar a sinerese e modificar a viscosidade e a textura de certos alimentos. Logo, a inulina e os probióticos podem ser utilizados em conjunto para a produção de queijo de cabra cremoso sem afetar negativamente as características gerais de qualidade do produto, e ainda agregar valor devido ao seu potencial simbiótico (BARBOSA *et al*., 2016).

O uso de inulina favoreceu a manutenção de *L. plantarum* em iogurte desnatado durante três semanas de armazenamento acima do limite estabelecido para o produto ser considerado probiótico (DELAVARI; POURAHMAD; SOKUTIFAR, 2014). Além disso, foram avaliados os efeitos da adição de inulina (1,5%) sobre a sobrevivência das bactérias probióticas *L. rhamnosus* e *L. reuteri* e em iogurte. Os resultados mostraram que a inulina afetou positivamente a sobrevivência dos probióticos e as qualidades sensoriais do iogurte simbiótico (SHAGHAGHI; POURAHMAD; ADELI, 2013). Queijos simbióticos também foram estudados. Os probióticos *L. acidophilus* La-5 e *B. animalis* Bb-12 foram adicionadas ao queijo petit-suisse e avalidas sob condições simuladas gastrointestinais *in vitro* (PADILHA *et al*., 2016). Foram fabricados dois lotes de queijo: um com bactérias probióticas, outro com inulina (7,5%) e FOS (2,5%). As estirpes probióticas apresentaram taxas de sobrevivência significativamente maiores no queijo simbiótico de petit-suisse no primeiro e no 28º dia de armazenamento a 4 °C (PADILHA *et al*., 2016). A adição destes prebióticos também proporcionou fermentação mais rápida e alta produção de ácido lático, promovendo maiores taxas de crescimento de bifidobactérias e lactobacilos, quando queijo tipo petit-suisse foi fabricado (CARDARELLI *et al*., 2007). Além disso, pastas de queijos contendo *Lactobacillus sakei* subsp. *sakei* e inulina apresentaram maior inibição de *L. monocytogenes* através da produção de bacteriocinas durante o armazenamento em relação aos queijos não simbióticos (MARTINEZ *et al*., 2015).

Quadro 6 – Exemplos de aplicação de bactérias probióticas em associação com prebióticos em derivados lácteos.

Produto	**Cepa probiótica**	**Prebiótico**	**Matéria-prima**	**Referências**
Queijo	*Enterococcus faecium* NRRL B-2354, *Bifidobacterium longum* NRRL B-41409, *Lactobacillus paracasei* subsp. *paracasei* NRRL B-4560	Inulina e oligofrutose	Leite de cabra	(KINIK *et al*., 2017)
Queijo cremoso	*Lactobacillus acidophilus* LA-05, *Bifidobacterium animalis* subsp *lactis* Bb-12	Inulina	Leite de cabra	(BARBOSA *et al*., 2016)
Queijo chevrotin	*Bifidobacterium lactis*	Inulina	Leite e leite de cabra	(BELTRAO *et al*., 2017)
Queijo fresco	*Bifidobacterium lactis* BB-12	Inulina	Leite crioconcentrado	(MUNOZ *et al*., 2017)
Queijo Mascarpone	*Bifidobacterium lactis* BB-12	Inulina	Creme de leite (30 g lipídeo/100 g)	(DE ALMEIDA *et al*., 2017)
Iogurte grego	*Bifidobacterium lactis* BB-12 microencapsulada	Inulina	Leite	(PINTO *et al*., 2017)
Iogurte de cupuaçu	*Lactobacillus acidophilus* LA-5	Inulina	Leite de cabra e cupuaçu	(COSTA *et al*., 2015)
Iogurte	*Lactobacillus acidophilus* L10 DSM, *Lactobacillus acidophilus* L10 NCFM, *Bifidobacterium animalis* subsp. *lactis* Bl04, *Bifidobacterium animalis* subsp. *lactis* HN019	Casca de maracujá em pó (estudada neste trabalho sob hipótese de ser um potencial prebiótico)	Leite em pó desnatado, leite em pó integral	(DO ESPÍRITO SANTO *et al*., 2012)

Produto	**Cepa probiótica**	**Prebiótico**	**Matéria-prima**	**Referências**
Iogurte	*Lactobacillus acidophilus, Bifidobacterium bifidium*	Lactulose, oligofrutose e inulina	Leite de búfala	(EHSANI *et al*., 2016)
Bebida láctea de chocolate	*Bifidobacterium lactis*	Mix de inulina e oligofrutose	Leite de cabra, soro de leite de cabra obtido da produção de queijo de coalho	(SILVEIRA *et al*., 2015)
Bebida láctea	*Lactobacillus Paracasei*	Inulina e oligofrutose	Leite	(FORNELLI *et al*., 2015)
Bebida láctea	*Bifidobacterium lactis* BB-12, *Lactobacillus acidophilus* LA-5	Oligofrutose	Leite e soro de leite proveniente da manufatura de queijo Minas Frescal	(DE CASTRO *et al*., 2008)
Bebida láctea	*Bifidobacterium lactis* BB-12, *Lactobacillus acidophilus* LA-5	Oligofrutose	Leite e soro de leite proveniente da manufatura de queijo Minas Frescal	(DE CASTRO *et al*., 2009)
Sorvete de pêssego	*Bifidobacterium lactis* BB-12	Inulina	Leite e pêssego	(VILLALVA *et al*., 2017)
Sorvete	*Bifidobacterium lactis* e *Lactobacillus casei* microencapsuladas (individualmente) com alginato de sódio e extrato de *Camellia sinensis.*	*Camellia sinensis* (planta estudada neste trabalho sob hipótese de ser um potencial prebiótico).	Leite e gordura de leite	(NOORI; KHAJI; GANDOMI, 2017)

Produto	**Cepa probiótica**	**Prebiótico**	**Matéria-prima**	**Referências**
Sobremesa láctea de chocolate	*Lactobacillus paracasei* subsp. *paracasei* LBC 81	Frutooligossacarídeo	Leite	(VALENCIA *et al*., 2016)
Sorvete	*Pediococcus pentosaceus UAM22*	Inulina	Leite e proteína do soro	(FRAGOSO *et al*., 2016)

Fonte: Os autores.

Outros prebióticos foram usados na elaboração de derivados lácteos funcionais com o objetivo de influenciar positivamente os probióticos. No que diz respeito às fórmulas para lactentes, a incorporação de 0,5% de lactulose foi considerada adequada para estimular o crescimento de bifidobactérias (NAGENDRA *et al*., 1995). Estudos de armazenamento para avaliar a estabilidade da fórmula infantil demonstraram que a adição de lactulose não influenciou a aceitabilidade do produto e não alterou qualquer característica de armazenamento (NAGENDRA; BASKARAN; RAO, 1995). Quando a lactulose é utilizada na fabricação de iogurte, várias propriedades funcionais podem ser melhoradas. O iogurte contendo lactulose foi eficaz no tratamento da constipação infantil, comparável ao iogurte contendo uma mistura de fibra alimentar constituída por transgalactooligosacarídeos, inulina, fibra de soja e amido resistente (KOKKE *et al*., 2008). Além disso, a adição de lactulose reduziu o período de incubação na fabricação iogurte contendo *Lactobacillus acidophilus* e *Bifidobacterium bifidum* e causou um aumento significativo na contagem celular de *B. bifidum* durante o armazenamento (ÖZER; AKIN; ÖZER, 2005).

A rafinose foi adicionada em leite fermentado e sua influência sobre a sobrevivência de *Bifidobacterium lactis* Bb-12 e *Lactobacillus acidophilus* La-5 forneceu evidências convincentes de que este prebiótico apresenta efeitos benéficos sobre a sobrevivência dessas culturas probióticas. Como resultado, o leite fermentado contendo probióticos e prebióticos mostrou ações sinérgicas na promoção da saúde (MARTÍNEZ-VILLALUENGA *et al*., 2006). Além disso, a suplementação com baixo teor de galactooligossacarídeos (0,24 g/100 mL) em fórmula infantil pode melhorar a frequência de fezes, diminuir o pH fecal e estimular bifidobactérias e lactobacilos (BEN *et al*., 2008).

Pesquisadores estão em constante busca por novos compostos prebióticos e suas possíveis combinações com os tradicionais probióticos, ou outros microrganismos desconhecidos que poderão ter sua eficácia comprovada. Como exemplo, o potencial uso de *Agave salmiana* como um prebiótico estimulador do crescimento de bactérias probióticas foi avaliado (MARTINEZ-GUTIERREZ *et al*., 2017). Pesquisas futuras podem otimizar o teor e o perfil dos frutooligossacarídeos presente nas plantas, através da seleção de variedades ou mudança de prática agronômica e pós-colheita, afim de desenvolver suas aplicações inovadoras para a indústria de alimentos e a promoção da saúde.

3 MICROENCAPSULAÇÃO DE PROBIÓTICOS

Diversas tecnologias foram avaliadas visando aumentar a resistência dos microrganismos probióticos contra condições adversas, e em consequência, melhorar a viabilidade probiótica em alimentos e no trato digestivo. Tais técnicas incluem a seleção de cepas resistentes a ácidos e à bile (LIONG; SHAH, 2005; JAYAMANNE; ADAMS, 2006; SHAHIDI *et al*., 2008), fermentação em duas etapas (LANKAPUTHRA; SHAH, 1997; TRUELSTRUP-HANSEN *et al*., 2002), uso de sistemas de embalagem apropriados (MILLER *et al*., 2002; WANG; YU; CHOU, 2004), adaptação ao estresse (MOZZETTI *et al*., 2012), inclusão de compostos protetores tais como proteína hidrolisada do soro de leite e oligossacarídeo (DABROWSKA *et al*., 2017; POURJAFAR *et al*., 2017), e microencapsulação (MACIEL *et al*., 2014; LIU *et al*., 2017; DIAS *et al*., 2018). A última opção é um dos métodos mais eficientes, e tem estado sob considerável investigação (MARTÍN *et al*., 2015).

3.1 DEFINIÇÃO DE MICROENCAPSULAÇÃO E O USO DE DIFERENTES TÉCNICAS

A microencapsulação pode ser definida como o processo no qual as células são retidas dentro de uma membrana encapsulante, visando reduzir a injúria de células ou a lise celular, de uma maneira que resulta na liberação apropriada de microrganismos no intestino. Algumas vantagens desta técnica são: conversão das células em uma forma de pó, facilitando o uso (uma vez que aumenta sua distribuição homogênea em todo o produto) e aumentando a versatilidade nas aplicações (MARTÍN *et al*., 2015); obtenção de microcápsulas com melhor estabiliade término (VERRUCK *et al*., 2018a); e melhora da sobrevivência do probiótico durante sua passagem pelo sistema gastrointestinal (DANTAS *et al*., 2021).

Várias técnicas de encapsulação de culturas probióticas foram e estão sendo estudadas por pesquisadores ao longo dos anos. As principais e mais investigadas se classificam em: extrusão (LIAO *et al*., 2019), secagem em leito fluidizado (SCHELL; BEERMANN, 2014), liofilização (MAO *et al*., 2018; ARCHACKA *et al*., 2019), *electrospinning* (LOPEZ-RUBIO *et al*., 2012; FENG *et al*., 2018), emulsificação (ALEHOSSEINI *et al*., 2018; ESHRATI *et al*., 2018), *spray chilling* (BAMPI *et al*., 2016; ARSLAN-TONTUL; ERBAS, 2017), e *spray drying* ou secagem por pulverização (LOYEAU *et al*., 2018; AREPALLY; GOSWAMI, 2019). A seleção de cada método é dependente da aplicação que será dada à microcápsula, do tamanho desejado, do mecanismo de liberação e das propriedades físico-químicas, tanto do material ativo, quanto do agente encapsulante.

Como mencionado acima, a literatura reporta diferentes alternativas tecnológicas objetivando melhorar a sobrevivência probiótica em produtos alimentícios, dentre elas, a

microencapsulação. Segundo Burgain *et al*. (2011), essa estratégia também ajuda a evitar o possível impacto sensorial negativo das bactérias livres quando incorporadas aos alimentos. O Quadro 7 expõe alguns produtos desenvolvidos adicionados de culturas probióticas previamente microencapsuladas.

Quadro 7 – Exemplos de probióticos encapsulados e suas aplicações em diferentes alimentos.

Produto	**Técnica de microencapsulação**	**Cultura(s) probiótica(s)**	**Agentes encapsulantes**	**Autores**
Suco de romã	Extrusão	*Lactobacillus plantarum* NCIMB 8826	Alginato de sódio e quitosana	(NUALKAEKUL *et al*., 2012)
Iogurte	Extrusão	*Lactobacillus acidophilus*	Xantana e quitosana	(SHU *et al.*, 2017)
Iogurte	Extrusão	*Enterococcus durans* 39C	Blendas poliméricas de alginato-psyllium com adição de inulina ou feno-grego	(HAGHSHENAS *et al*.,2015)
Iogurte de leite de cabra	Extrusão	*Bifidobacterium animalis* subsp *lactis* BB-12	Matriz composta de leite de cabra, alginato de sódio e inulina	(PRASANNA; CHARALAMPOPOULOS, 2019)
Iogurte e suco de laranja	Extrusão	*Lactobacillus acidophilus* LA 5 e *Lactobacillus casei* LC 01	Alginato de sódio, inulina e galactooligossacarídeos	(KRASAEKOOPT; WATCHARAPOKA, 2014)
Chocolate	Extrusão seguida de liofilização	*Akkermansia muciniphila* DSM22959 (sugerido pelos autores como potencial probiótico) e *Lactobacillus plantarum* subsp. *plantarum* ATCC14917	Goma xantana e goma gelana	(MARCIAL-COBA *et al*., 2018)
Sorvete	Emulsificação	*Lactobacillus casei* Lc-01 e *Bifidobacterium lactis* Bb-12	Alginato de cálcio	(HOMAYOUNI *et al*., 2008)

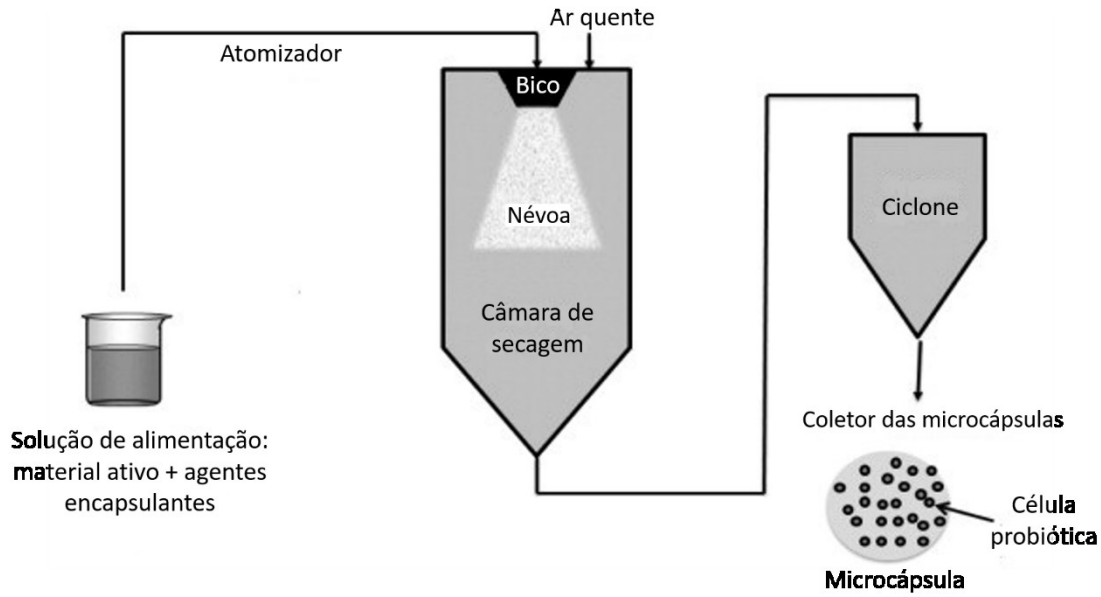
Produto	**Técnica de microencapsulação**	**Cultura(s) probiótica(s)**	**Agentes encapsulantes**	**Autores**
Queijo muçarela	Emulsificação	*Lactobacillus paracasei* LBC-1e	Alginato de sódio	(ORTAKCI *et al*., 2012)
Frozen	Emulsificação seguido de gelificação	*Lactobacillus acidophilus* La-5	Alginato de cálcio	(AHMADI *et al*., 2014)
Sorvete	Emulsificação seguido de gelificação	*Lactobacillus casei* e *Bifidobacterium lactis* (microencapsuladas separadamente)	Alginato de sódio e extrato de *Camellia sinensis*	(NOORI; KHAJI; GANDONI, 2017)
Queijo fresco	Emulsificação seguido de gelificação	*Lactobacillus rhamnosus* 6134	Proteína de soro de leite concentrada (WPC-80) e isomaltooligosacarídeos	(LIU *et al*., 2017)
Iogurte	Gelificação iônica	*Lactobacillus acidophilus* LA-5	Alginato, gelatina tipo A e frutooligossacarídeo	(SILVA *et al*., 2018)
Pão	Liofilização	*Lactobacillus plantarum* P8	Leite desnatado reconstituído, goma arábica, maltodextrina e inulina	(ZHANG *et al*., 2018)
Chocolate	*Spray-coating*	*Lactobacillus helveticus* e *Bifidobacterium longum*		(POSSEMIERS *et al*., 2010)

Produto	**Técnica de microencapsulação**	**Cultura(s) probiótica(s)**	**Agentes encapsulantes**	**Autores**
Chocolate	*Spray-coating*	*Lactobacillus helveticus* e *Bifidobacterium longum*	Ácidos graxos	(MAILLARD; LANDUYT, 2008)
Suco de cenoura	*Spray drying* seguido de liofilização	*Lactobacillus casei*-01	Alginato de cálcio, quitosana e frutooligossacarídeo	(IVANOVSKA *et al*., 2014)
Queijo tipo Gouda	*Spray drying*	*Bifidobacterium lactis* BLC1	Complexo de β-ciclodextrina e goma arábica ou leite desnatado reconstituído	(BORRAS-ENRIQUEZ *et al*., 2018)
Creme de ricota	*Spray drying*	*Bifidobacterium* BB-12	Leite desnatado reconstituído, inulina e oligofrutose	(FRITZEN-FREIRE *et al*., 2013)
Iogurte estilo grego	*Spray drying*	*Bifidobacterium animalis* subsp *lactis* BB-12	Soro de leite doce ou soro de leite doce e inulina	(PINTO *et al*., 2017)
Mousse	*Spray drying*	*Lactobacillus acidophilus* La-5	Inulina, leite desnatado e frutooligossacarídeo	(Dos SANTOS *et al*., 2019)
Pudim	*Spray drying*	*Lactobacillus casei* Shirota	Diferentes combinações de maltodextrina, leite desnatado reconstituído e goma arábica	(GUL, 2017)

Fonte: A autora.

Segundo Gul (2017), a secagem por *spray drying* é o método de microencapsulação mais utilizado devido ao seu baixo custo, sua rápida produção de grande quantidade de células viáveis, e tecnologia bem estabelecida. A Figura 6 mostra um esquema dessa técnica: a solução de alimentação é pressurizada e logo em seguida atomizada, formando uma "névoa" na câmara de secagem. O gás quente (ar ou nitrogênio) também é injetado na câmara de secagem, permitindo a evaporação do solvente. Por fim, as microcápsulas são separadas do ar quente por um ciclone e coletadas na parte inferior (BURGAIN *et al*., 2011).

Figura 6 – Representação esquemática da microencapsulação no equipamento *spray dryer*.



Fonte: Adaptado de Burgain *et al*. (2011).

A eficiência na retenção do material ativo no núcleo está relacionada à parâmetros do processo (temperatura de secagem e tamanho de gotícula formada), características do agente encapsulante (tamanho das moléculas, solubilidade) e características do material ativo (polaridade, pressão de vapor, tamanho de molécula) (RÉ, 1998). Nesse contexto, a escolha do(s) agente(s) encapsulante(s) (também chamado material de parede, membrana, fase externa, suporte ou matriz) é muito importante (GHARSALLAOUI *et al*., 2007). Dentre os agentes encapsulantes mais testados (sozinhos ou em associações), destacam-se os oligossacarídeos prebióticos (fructooligossacarídeos, galactooligossacarideos, isomaltooligossacarídeos, e xilooligossacarídeos); polissacarídeos prebióticos (inulina); biopolímeros diversos, como goma arábica (GA), alginatos e carragenas; leite em pó integral ou desnatado reconstituídos (LIR e LDR, respectivamente); proteínas do soro de leite; maltodextrinas; amidos; e gelatina (PINTO

et al., 2015; VERRUCK *et al*., 2017; SOHAIL *et al*., 2013; LIÃO *et al*., 2019; MAO *et al*., 2019; GUL, 2017).

3.2 AGENTES ENCAPSULANTES

Segundo Haffner, Diab e Pasc (2016), matrizes como proteínas naturais e polissacarídeos são amplamente utilizadas na produção de pós probióticos devido à sua origem natural, não-toxicidade e biodegradabilidade. Como visto no Quadro 7, são vários os agentes encapsulantes investigados. No presente tópico, abordaremos mais detalhadamente o leite e os prebióticos.

3.2.1 Leite desnatado reconstituído (LDR)

O LDR tem sido comumente utilizado como agente encapsulante, mostrando um efeito favorável na manutenção ou aumento da sobrevivência microbiana durante o processo de *spray drying* (FU; CHEN, 2011; PAÉZ *et al*., 2012). Esse agente é resultante da solubilização em água do leite desnatado em pó.

A encapsulação em LDR foi tão boa ou melhor em comparação com a maltodextrina e inulina (ZHANG *et al*., 2018; GUL, 2017), e β-ciclodextrina e goma arábica (BORRAS-ENRIQUEZ *et al*., 2018; GUL, 2017). Fritzen-Freire *et al*. (2013), Maciel *et al*. (2014) e Archacka *et al*. (2019) também obtiveram sucesso ao empregar LDR para microencapsular – via técnica de *spray drying* – *Bifidobacterium* BB-12, *Lactobacillus acidophilus* La-5 e *Lactococcus lactis*, respectivamente.

Diversos autores sugerem que a eficiência da matriz láctea em proteger a viabilidade celular durante a secagem está relacionada à influência dos componentes do leite (ANANTA; VOLKERT; KNORR, 2005; FU; CHEN, 2011). O perfil de proteínas do leite desnatado reconstituído se constitui principalmente de caseínas (FOX; McSWEENEY, 1998). Segundo Khem, Small e May (2016), o leite desnatado exerce o seu efeito protetor sobre as células devido à produção de uma camada de revestimento na superfície celular. Essa camada se forma durante um curso de interações hidrofóbicas entre as células e as proteínas do leite, o que resulta na estabilização da membrana celular. Além disso, as proteínas podem interagir com o cálcio do leite, o que auxilia na formação do revestimento protetivo na parede da célula bacteriana (HUANG *et al*., 2014; ZHENG *et al*., 2016). O cálcio pode também causar agregação da proteína durante tratamentos térmicos, como mostra o trabalhao de Wang *et al*. (2016), no qual

verifica-se que a proteção do *Lb. Rhamnosus* GG foi maior no leite desnatado que passou por secagem convectiva de gotículas (*single droplet drying*) do que naquele que não foi submetido ao aquecimento. Acredita-se que esta proteção melhorada esteja ligada à estrutura gelatinosa da proteína agregada ao cálcio, que exibe rápida cinética de secagem semelhante à água e forma uma partícula porosa. Guerin *et al*. (2017) desenvolveram microcápsulas inovadoras obtidas pelo processo de *spray drying*: diferentes comportamentos de reconstituição foram aprimorados com base na agregação de proteína do leite controlada por quimosina.

Por outro lado, Morgan *et al*. (2006) comentam que uma mistura de proteínas e açúcares parece ser o veículo mais efetivo na proteção de microrganismos durante a secagem. Essa observação é chancelada através dos estudos de Sadguruprasad e Basavaraj (2018), no qual testou-se a viabilidade de cepas de *Lactobacillus acidophilus* quando as mesmas foram liofilizadas em uma suspensão contendo sacarose (1,2%) e leite desnatado reconstituído (6%). Esse meio de suspensão foi significativamente eficaz na manutenção de alto grau de sobrevivência das células durante 2 anos de armazenamento a 4 °C. Marcial-Coba *et al.* (2018) verificaram também que apenas soluções crioprotetoras com alto teor de açúcar ou proteína melhoraram significativamente a viabilidade de *Akkermansia muciniphila* e *Lactobacillus plantarum* durante a liofilização.

Silva *et al*. (2011) relatam que a proteção conferida pelo LDR é dependente do microrganismo utilizado e dos parâmetros empregados no *spray dryer*. Além disso, o tipo de agente encapsulante pode não só afetar a sobrevivência das bactérias durante o processamento, prolongar o seu prazo de validade e influenciar a sua resistência às condições do trato gastrointestinal, mas também afetar significativamente a capacidade das células probióticas de se aderirem ao epitélio intestinal. Esta última característica foi constatada no trabalho de Archacka *et al*. (2019), onde se observou um aumento de 25x no número de *L. lactis* aderidos ao epitélio quando estes foram microencapsulados em LDR do que quando a matriz foi o dissacarídeo trealose. Em contrapartida, a substituição parcial do LDR por outros agentes encapsulantes, como os prebióticos, poderia aumentar a proteção dos microrganismos tanto durante o *spray drying*, como durante armazenamento e também sob condições gastrointestinais simuladas (FRITZEN-FREIRE *et al*., 2012; FRITZEN-FREIRE *et al*., 2013).

3.2.2 Prebióticos

A inulina como matriz de encapsulação tem sido amplamente utilizada para proteger as células probióticas e melhorar sua sobrevivência durante a secagem pelo método de *spray*

drying (BUSTAMANTE *et al*., 2015). Devido ao seu alto grau de polimerização, é termicamente estável e pouco solúvel (WADA *et al*., 2005). Nunes *et al*. (2018) observaram que a inulina e milho quando utilizados como agentes encapsulantes de *L. acidophilus* La-5 apresentaram maior eficiência de encapsulação em comparação com goma arábica e trealose. Além disso, a inulina é um prebiótico comumente utilizado em produtos lácteos e tem demonstrado aumentar a viabilidade de bifidobactérias no intestino grosso (NAZZARO *et al*., 2012). Nesse contexto, trata-se de um ingrediente que pode ser utilizado tanto como agente encapsulante bem como alimento prebiótico.

Alguns estudos relatam também o uso de frutooligossacarídeos como material de parede em processos de *spray drying* (VERRUCK *et al*., 2017; IVANOVSKA *et al*., 2014; RAJAM; ANANDHARAMAKSHNAN, 2015). No trabalho de Silva *et al*. (2018), o uso de frutooligossacarídeo como agente encapsulante melhorou a viabilidade de *L. acidophilus* em iogurte, uma vez que atuou como fonte de substrato e promoveu a formação de uma matriz de encapsulação mais resistente à dissolução quando submetida a condições gastrointestinais. Ou seja, o uso desse prebiótico favoreceu a liberação lenta e controlada do probiótico ao longo do trato gastrointestinal, indicando, portanto, uma aplicação promissora das microcápsulas simbióticas. Por outro lado, o comportamento gomoso limita a aplicação dos frutooligossacarídeos, devido principalmente à sua menor temperatura de transição vítrea (se comparados a polissacarídeos). No entanto, a gomosidade durante a secagem por *spray drying* pode ser minimizada alterando a temperatura de transição vítrea pela introdução de agentes de alto peso molecular (ADHIKARI *et al*., 2009). Por exemplo, Biedrzycka e Bielecka (2004) sugerem o uso de misturas de oligofrutose e inulina, aliado ao fato de que a oligofrutose atua mais intensamente na região proximal do cólon, enquanto a inulina é mais efetiva na parte distal. Além disso, outros relatos sugeriram que as microcápsulas simbióticas são mais eficazes quando expostas ao sistema gastrointestinal simulado (CHÁVARRI *et al*., 2010; COOK *et al*., 2014).

3.3 CARACTERIZAÇÃO DAS MICROCÁPSULAS OBTIDAS POR *SPRAY DRYING*

A utilização de diferentes agentes encapsulantes na produção de microcápsulas pelo método de *spray drying* pode resultar em pós com diferentes propriedades físicas (DIAS *et al*., 2018). Segundo Verruck *et al*. (2018b), a caracterização e o monitoramento das microcápsulas são abordagens interessantes que visam melhorar e determinar as condições ótimas ao adicioná-las a produtos alimentícios.

3.3.1 Umidade, atividade de água, e sorção de umidade

Meng *et al*. (2008) ressaltam a importância do controle da temperatura de saída das microcápsulas da câmara de secagem do equipamento *spray dryer*, já que este é o parâmetro que mais influencia na taxa de sobrevivência dos microrganismos durante o processo. Além disso, em um processo de secagem, a temperatura do ar tem efeito significativo sobre a atividade de água (a_w) do produto em pó (COSTA *et al*., 2015).

A a_w de alimentos é um conceito termodinâmico de equilíbrio. É definido como a razão entre a pressão de vapor da água em equilíbrio com o alimento à determinada temperatura e a pressão de vapor da água pura à mesma temperatura. Assim, a a_w indica como a água é ligada aos alimentos e, portanto, sua disponibilidade para participar de reações físicas, químicas e microbiológicas (AL-MUHTASEB; MCMINN; MAGEE, 2002). O teor de umidade em torno de 4 g 100 g^{-1} é geralmente recomendado para manutenção adequada da viabilidade de probióticos em microcápsulas, e também para evitar a aglomeração durante armazenamento prolongado (HEIDEBACH; FORST; KULOZIK, 2010; SCHUCK, 2011).

Quando um material biológico é exposto ao ambiente com determinada umidade relativa, este tende a ajustar sua umidade até a condição de equilíbrio com o ambiente. Sendo assim, para muitas substâncias e ingredientes de alimentos, é de grande valia conhecer as propriedades de adsorção de água e sua cinética. A cinética de adsorção define seu comportamento físico e químico, bem como a vida útil de muitos produtos alimentícios. Portanto, é extremamente importante conhecer essas propriedades ou pelo menos saber como mensurá-las e modelá-las com precisão (PAQUET-DURAND; ZETTEL; HITZMANN, 2015). O leite em pó, por exemplo, é desidratado principalmente para prolongar sua vida útil e reduzir os custos de transporte. Normalmente é utilizado reidratado, logo, é fundamental conhecer a duração do processo de hidratação desse leite, bem como saber a maneira correta de armazená-lo para que não faça trocas gasosas com o meio ambiente (SHRESTHA *et al*., 2007).

No processo de sorção, as moléculas de água se distribuem entre a fase vapor e a superfície do alimento até atingir um estado de equilíbrio (RHIM; KOH; KIM, 2011). Esse fenômeno de sorção pode ser estudado pela mensuração da taxa de aproximação ao equilíbrio, no caso, por meio de curvas de adsorção, que por sua vez, são gráficos que representam a massa em função do tempo a uma dada umidade relativa e temperatura constante. O modelo empírico proposto por Peleg se destaca quando aplicado ao fenômeno de adsorção de alimentos. Peleg propôs uma equação não exponencial que descreve a adsorção de água no arroz e no leite em pó, onde k_2 é inversamente relacionado à umidade de equilíbrio e k_1 é inversamente relacionado

à taxa inicial de adsorção de água. Uma das principais vantagens do modelo Peleg é economizar tempo, prevendo a cinética de sorção da água dos alimentos usando apenas alguns pontos experimentais (PELEG, 1988). Segundo Paquet-Durand, Zettel e Hitzmann (2015), para analisar a adsorção de água usando o modelo Peleg, as medições experimentais devem ser realizadas com a maior precisão possível para que os parâmetros do modelo sejam determinados. Além disso, esse modelo é amplamente utilizado devido à sua relativa simplicidade, ao bom planejamento experimental e às suas moderadas necessidades computacionais (PAQUET-DURAND; ZETTEL; HITZMANN, 2015).

3.3.2 Análises térmicas

As análises térmicas representam uma série de técnicas analíticas que medem as propriedades físicas e químicas dos alimentos em função da temperatura, tempo e atmosfera (gás inerte ou oxidante, pressão e umidade relativa). Nessas técnicas, a temperatura é geralmente programada para aumentar ou diminuir a uma taxa linear. Uma vez que a temperatura e o tempo são controlados em todas as etapas de produção de alimentos, os instrumentos de análise térmica podem simular esses processos em uma escala muito pequena (miligramas) e medir a resposta do material (THOMAS; SCHMIDT, 2010). Portanto, de acordo com Thomas e Schmidt (2010), os resultados da análise térmica fornecem informações sobre a estrutura e a qualidade da matéria-prima e dos produtos finais. As características destes, como textura e estabilidade ao armazenamento, são influenciadas pela estrutura física (amorfa, cristalina, semicristalina) da matéria-prima utilizada. As áreas de aplicação das análises térmicas incluem garantia de qualidade, desenvolvimento de produtos e pesquisa de novos materiais, formulações e condições de processamento. As técnicas de análise térmica mais frequentemente utilizadas na pesquisa de alimentos incluem calorimetria diferencial de varredura (DSC) e análise termogravimétrica (TGA) (BILIADERIS, 1983).

Na técnica de DSC, as mudanças nas propriedades físicas ou químicas dos materiais em função da temperatura são monitoradas através da detecção das mudanças de calor envolvidas nesses processos (IONASHIRO, 2004). Nesta técnica, o princípio de medição é comparar as taxas de fluxo de calor da amostra com as de um material inerte, que é então aquecido ou resfriado na mesma taxa (BILIADERIS, 1983). Quando ocorrem mudanças na estrutura da amostra (ou seja, transição), há absorção ou liberação de calor; em consequência, o fluxo de calor diferencial é registrado como um pico (THOMAS; SCHMIDT, 2010). A área sob esse pico é diretamente proporcional à mudança entálpica, e sua direção indica se o evento

térmico é endotérmico ou exotérmico (BILIADERIS, 1983). A principal aplicação da DSC em ingredientes lácteos é determinar várias transições termodinâmicas de fase/estado, incluindo transição vítrea, cristalização, fusão e desnaturação. Além disso, durante o processamento e armazenamento, podem ocorrer alterações nos estados físicos dos componentes do leite (lactose, gorduras, proteínas, água e minerais), resultando em variações na funcionalidade e estabilidade do produto. Na maioria dos casos, essas alterações estão relacionadas às transições de fase de primeira ou segunda ordem, sendo a última a transição vítrea, enquanto a primeira pode estar relacionada à fusão e/ou cristalização de solutos como lactose, como também à desnaturação de proteínas. Portanto, para uma melhor compreensão das propriedades e estabilidade dos ingredientes lácteos durante o processamento e armazenamento, a DSC pode ser aplicada visando conhecer os estados físicos e as transições de fase em dadas condições (ZHOU; LABUZA, 2011).

A TGA fornece informações sobre a composição (número de componentes) do material e sua estabilidade térmica ou oxidativa (decomposição em atmosferas inertes e oxidantes, respectivamente). Essa análise deve ser a primeira técnica de análise térmica usada para caracterizar um novo material (THOMAS; SCHMIDT, 2010). Os instrumentos TGA usam uma balança analítica especialmente projetada e muito sensível para medir as alterações de massa, pois a amostra é tipicamente aquecida da temperatura ambiente para 1000 °C ou mais (IONASHIRO, 2004). Um termopar localiza-se próximo à amostra para registrar continuamente a temperatura conforme ocorrem alterações de peso. Um gás flui através da balança e cria uma atmosfera que pode ser inerte, nesse caso, nitrogênio, argônio ou hélio; oxidante, como ar ou oxigênio; ou redutora, por exemplo, gás formado de 8 - 10% de hidrogênio em nitrogênio (PRIME *et al*., 2009). A maioria das alterações de massa se constitui em sua diminuição, devido à volatilização ou decomposição. Todavia, o aumento de massa é observado durante os estágios iniciais de oxidação dos componentes dos alimentos. Assim, a TGA é uma excelente ferramenta para determinar a composição dos materiais, bem como a temperatura na qual esses materiais se decompõem devido à degradação térmica ou oxidativa (THOMAS; SCHMIDT, 2010).

4 CRIOCONCENTRAÇÃO

A crioconcentração é uma operação unitária de separação utilizada para concentrar líquidos por meio do congelamento e posterior separação de uma fração de água congelada. O

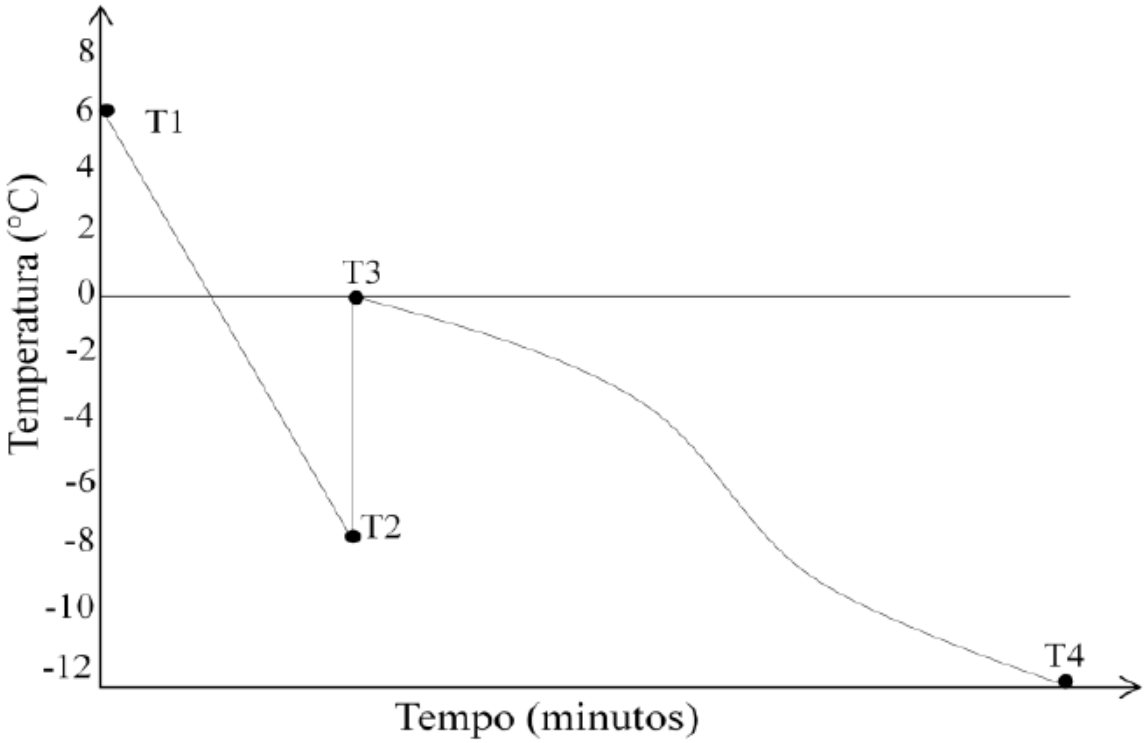
processo consiste basicamente na redução controlada da temperatura da solução de interesse, abaixo do seu ponto de congelamento, com o intuito de evitar a temperatura eutética no sistema onde todos os componentes da solução se apresentam como sólido (RAVENTÓS *et al*., 2007). Ao reduzir a temperatura da solução inicia-se o processo de cristalização da água, o que permite a formação e separação de cristais, dando origem a um produto concentrado (BELÉN *et al*., 2012; SÁNCHEZ *et al*., 2009). Um sistema de crioconcentração básico compreende duas etapas fundamentais: congelamento da solução e separação de uma fração concentrada e outra de cristais de gelo.

A etapa de cristalização entende-se também como um processo de transferência de calor e massa que ocorre devido a diferença de temperatura entre o fluído e o sistema de resfriamento. Observa-se uma trasnferência de calor entre o fluído a ser concentrado e a placa refrigerante, ao mesmo tempo que ocorre a transferência de solutos entre a fração de gelo e a solução a ser concentrada. Em um sistema de crioconcentração observam-se três etapas básicas: formação de uma estrutura cristalina, crescimento dos cristais de gelo, e reorganização da estrutura cristalina. A forma com que a estrutura cristalina é formada irá determinar o tamanho e a distribuição dos cristais de gelo. A velocidade de congelamento é o parametro utilizado para controlar o crescimento destes cristais em um sistema de crioconcentração (PETZOLD; AGUILERA, 2013). Após a formação dos cristais de gelo, o seu crescimento é controlado pela taxa de calor liberada durante a mudança de fase da solução (líquido-sólido) e também pela velocidade de transferência de massa. A solução é concentrada ao mesmo tempo que o volume dos cristais aumenta e a temperatura do sistema diminui. Por fim, os cristais de gelo formados se reorganizam em número, tamanho, forma e orientação (CHEN; CHEN; FREE, 1998; SÁNCHEZ *et al*., 2009)

Durante os processos de concentração de alimentos líquidos pelo frio, geralmente se obtém um comportamento de resfriamento semelhante para distintas amostras. A Figura 7 apresenta um gráfico adaptado da curva de resfriamento de suco de laranja concentrado por crioconcentração (SÁNCHEZ, 2011). Através desta curva é possivel identificar os principais pontos de transição de fase da solução. O ponto T1 representa a temperatura inicial da solução. O intervalo entre os pontos T1 e T2 é chamado de subresfriamento, onde a temperatura cai abaixo do seu ponto de congelamento, sem a formação de cristais de gelo. O ponto T2 indica o início da nucleação. O ponto T3 corresponde ao início da cristalização e ponto de congelamento da solução, onde se observa um aumento da temperatura associado ao calor gerado para formar os primeiros cristais de gelo (calor latente). Quanto mais solutos possui o alimento, menor é a temperatura de T3. Após atingir a temperatura T3, os cristais de gelo começam a crescer e a

solução a crioconcentrar. A medida que os cristais crescem e a solução é concentrada, a temperatura do sistema começa a cair novamente até chegar ao ponto T4, que corresponde à temperatura do meio refrigerante (por exemplo, se uma amostra foi submetida ao congelamento num congelador que opera à – 20 ºC, o ponto T4 será – 20 ºC). Na temperatura de T4, soluto e solvente se cristalizam; logo, não há mais separação de uma fase concentrada. Em suma, todos os alimentos líquidos apresentam este perfil de resfriamento, contudo, os pontos de nucleação e congelamento são diferentes para cada alimento.

Figura 7 – Curva adaptada de resfriamento de suco de laranja.



Fonte: Adaptado de Sánchez (2011).

4.1 SISTEMAS DE CRIOCOCENTRAÇÃO

Existem diferentes sistemas de crioconcentração que basicamente se diferem pelos seus mecanismos de formação dos cristais de gelo. No âmbito da concentração de fluídos alimentares podemos citar três técnicas: crioconcentração em suspensão, crioconcentração progressiva, e crioconcentração em bloco (também chamada de crioconcentração por congelamento-descongelamento) (AIDER; OUNIS, 2012; SÁNCHEZ *et al*., 2009).

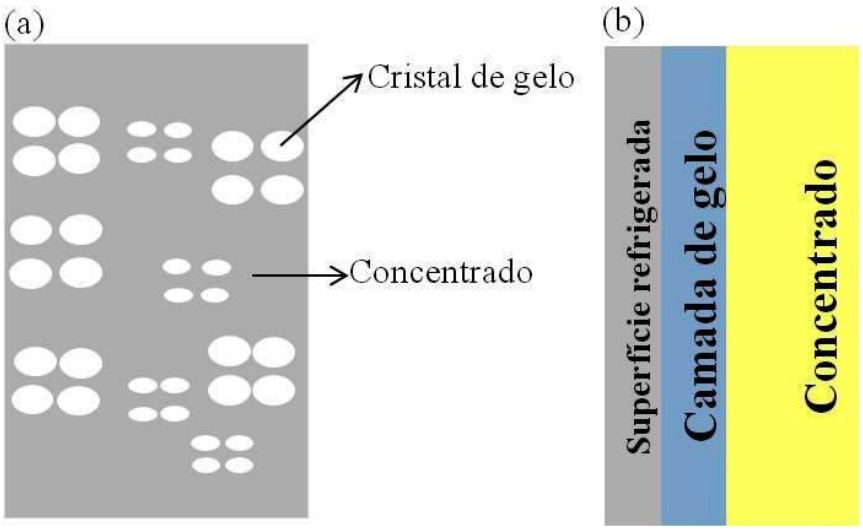
4.1.1 Crioconcentração em suspensão

O sistema é composto de uma fase inicial de geração de vários cristais de gelo (nucleação) seguido de uma fase de crescimento dos núcleos de gelo na solução, o que leva a uma cristalização parcial da água (Figura 8a). A separação dos cristais de gelo a partir da solução concentrada é crucial para o processo, no entanto o tamanho dos cristais de gelo ainda é limitado (MIYAWAKI *et al*., 2005; SÁNCHEZ *et al*., 2009). Esta técnica é considerada eficiente em termos de pureza da fração de gelo e aumento da concentração (QIN *et al*., 2006). Contudo, é necessário um sistema complexo de separação dos cristais gelo, o que provoca um substancial aumento nos custos de operação (MIYAWAKI *et al*., 2005; SÁNCHEZ *et al*., 2009).

4.1.2 Crioconcentração progressiva

Diferente do que acontece na crioconcentração em suspensão, nesta técnica ocorre a formação de um único cristal de gelo em contato com uma superfície refrigerada (Figura 8b). Desta maneira a separação do cristal de gelo se torna muito mais fácil, podendo ser realizada no mesmo equipamento (RAVENTÓS *et al*., 2007).

Figura 8 – Processo de cristalização em suspensão (a) e cristalização em película (b).



Fonte: A autora.

Este sistema pode apresentar-se em um equipamento tubular ou vertical. O sistema tubular consiste basicamente de dois tubos concêntricos conectados onde circulam a solução a ser concentrada e o fluido refrigerante. Já o sistema vertical compreende um tanque encamisado cilíndrico contendo um fluído refrigerante. A solução a ser concentrada é colocada no interior

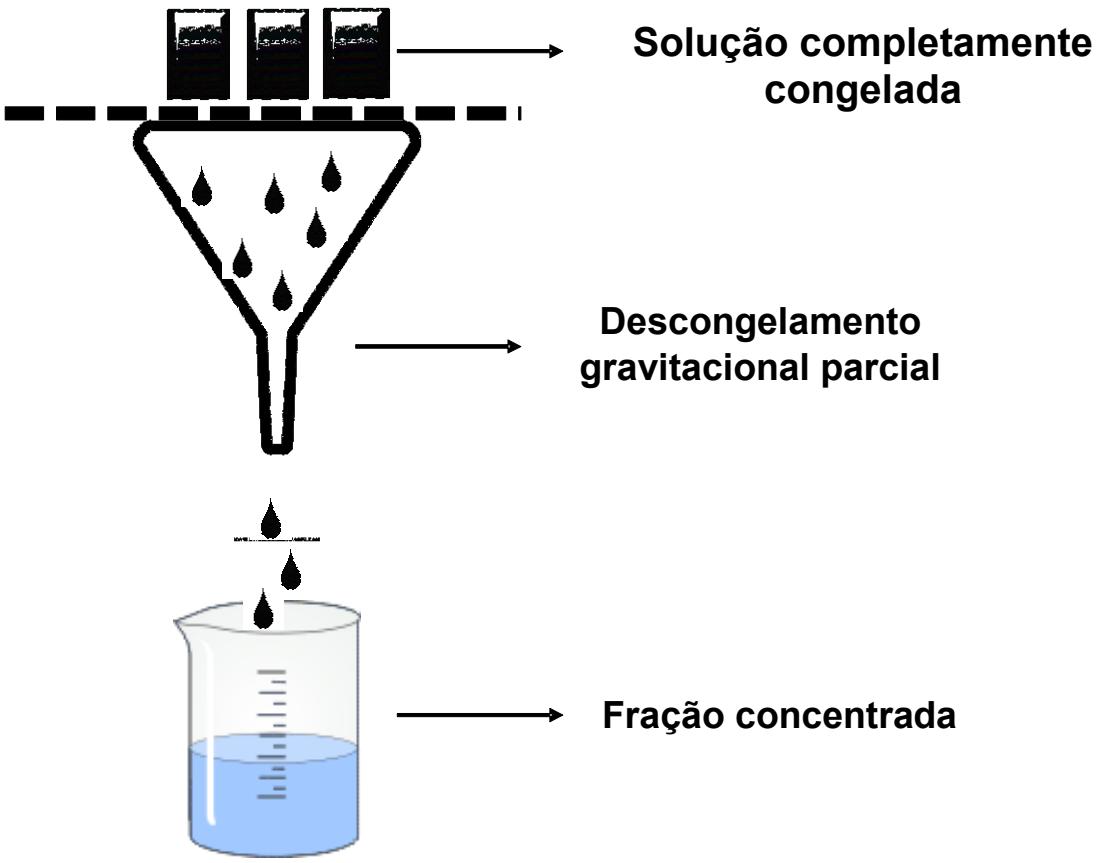
do cilindro e o gelo cresce aderido à parede do cilindro. O emprego de agitação mecânica auxilia na diminuição da retenção de sólidos na fração de gelo. Alguns parâmetros são primordiais para reduzir a retenção de sólidos na camada de gelo, como por exemplo, o fluxo da solução, sua concentração inicial, e temperatura de resfriamento (MIYAWAKI *et al*., 2005; OJEDA *et al*., 2017).

4.1.3 Crioconcentração em bloco

O emprego desta técnica consiste no congelamento total da solução seguido de um descongelamento parcial. Neste processo de descongelamento há a recuperação da fração líquida concentrada (Figura 9). Assim, a crioconcentração em bloco é composta por três etapas: congelamento, descongelamento e separação das frações (concentrado e gelo). O descongelamento gravitacional pode ser assistido por outras técnicas, a fim de melhorar a eficiência da separação (AIDER; DE HALLEUX, 2008; PETZOLD *et al*., 2015). Por exemplo, vácuo e centrifugação já foram investigados.

Uma das vantagens deste método é a possibilidade de realizar vários ciclos, o que favorece o aumento da concentração (AIDER; OUNIS, 2012). É uma tecnologia promissora, sendo eficaz na produção de alimentos líquidos concentrados com um elevado valor nutritivo e sensorial (AIDER; DE HALLEUX, 2009). Contudo, são necessárias pesquisas relacionadas à diminuição da retenção de sólidos na fração de gelo para alcançar as condições industriais adequadas.

Figura 9 – Esquema básico de crioconcentração em bloco.



Fonte: A autora.

4.2 EFICIÊNCIA DO PROCESSO E APLICAÇÕES NA ÁREA DE ALIMENTOS

A eficiência desta técnica é determinada em função da pureza do gelo obtido e também através do seu consumo energético (AIDER; OUNIS, 2012; PAZMIÑO *et al*., 2016; PETZOLD *et al*., 2015). Sabe-se que o consumo energético de um sistema de crioconcentração tende a ser menor do que no sistema de evaporação, dado o menor calor latente de solidificação da água (335 kJ/kg) em comparação com o calor latente de evaporação (2260 kJ/ kg) (JUSOH; MOHD YOUNS; ABU HASSAN, 2008). Desta forma, para alcançar os maiores níveis de eficiência é necessário encontrar as condições termodinâmicas ótimas, através da compreensão dos processos de transferência de calor e massa que ocorrem durante a crioconcentração. A maior dificuldade está em controlar a velocidade de crescimento dos cristais de gelo para evitar a retenção de sólidos no mesmo. Além disso, é importante estudar a natureza da solução a ser concentrada e entender os fenômenos que ocorrem durante o processo. Assim, os seguintes fatores devem ser avaliados: concentração inicial da solução, ponto de congelamento,

viscosidade da solução de alimentação, temperatura de resfriamento, e agitação mecânica (OJEDA *et al*., 2017).

Aider e Ounir (2012), que concentraram leite desnatado pelo processo de crioconcentração em bloco, observaram que após dois ciclos de crioconcentração, a viscosidade da solução aumentou significativamente resultando na diminuição da eficiência do processo. Estes autores constataram que o aumento da viscosidade da solução influencia as propriedades de cristalização. Além disso, observou-se que as taxas de crescimento e maturação dos cristais de gelo são afetadas pela presença de componentes como a lactose, que atua como um agente crio-protetor, prejudicando assim a separação (BLANQUET *et al*., 2005). Comportamento semelhante já havia sido observado por Aider, Halleux e Akbache (2007) ao concentrar soro de leite por crioconcentração.

Para a indústria de derivados lácteos, o emprego da crioconcentração se torna interessante em termos da manutenção de sabores específicos no leite concentrado. O leite pode ser facilmente concentrado através da técnica de evaporação, contudo, Aider e Ounis (2012) discutiram que a sua estabilidade é afetada em temperaturas acima de 70°C, podendo ocasionar a agregação irreversível de proteínas sensíveis ao calor. Ao utilizar o método de crioconcentração em substituição às técnicas que empregam calor para concentrar, seria possível diminuir os danos aos compostos termossensíveis presente no leite e ainda promover o aumento do teor de proteínas e lactose, agregando valor à matéria-prima e diversificando a produção no setor lácteo (AIDER; OUNIS, 2012).

O emprego desta tecnologia assegura maior qualidade do produto concentrado (AIDER; DE HALLEUX, 2009), proporcionando a remoção mais seletiva da água do que a evaporação ou processo de separação por membranas, como a microfiltração e a ultrafiltração (YEE; WILEY; BAO, 2007). Por fim, verifica-se a aplicação desta tecnologia em soro de leite (AIDER; DE HALLEUX; AKBACHE, 2007; DE LIZ *et al*., 2020), leite e leite de cabra (MUÑOZ *et al*., 2018; MACHADO CANELLA *et al*., 2020), suco de frutas (SÁNCHEZ *et al*., 2009; PETZOLD *et al*., 2015) e vinho (PETZOLD *et al*., 2016). Do ponto de vista de valorização, novas opções de utilização do leite concentrado devem ser levadas em consideração, como por exemplo, na produção de queijos frescos.

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CAPÍTULO 2

Lactose-free skim milk and prebiotics as carrier agents of *Bifidobacterium* BB-12 microencapsulation: physicochemical properties, survival during storage and *in vitro* gastrointestinal condition behaviour

*** Trabalho publicado (redação na íntegra)**

International Journal of Food Science and Technology 2020

Original article

Lactose-free skim milk and prebiotics as carrier agents *of Bifidobacterium* BB-12 microencapsulation: physicochemical properties, survival during storage and *in vitro* gastrointestinal condition behaviour

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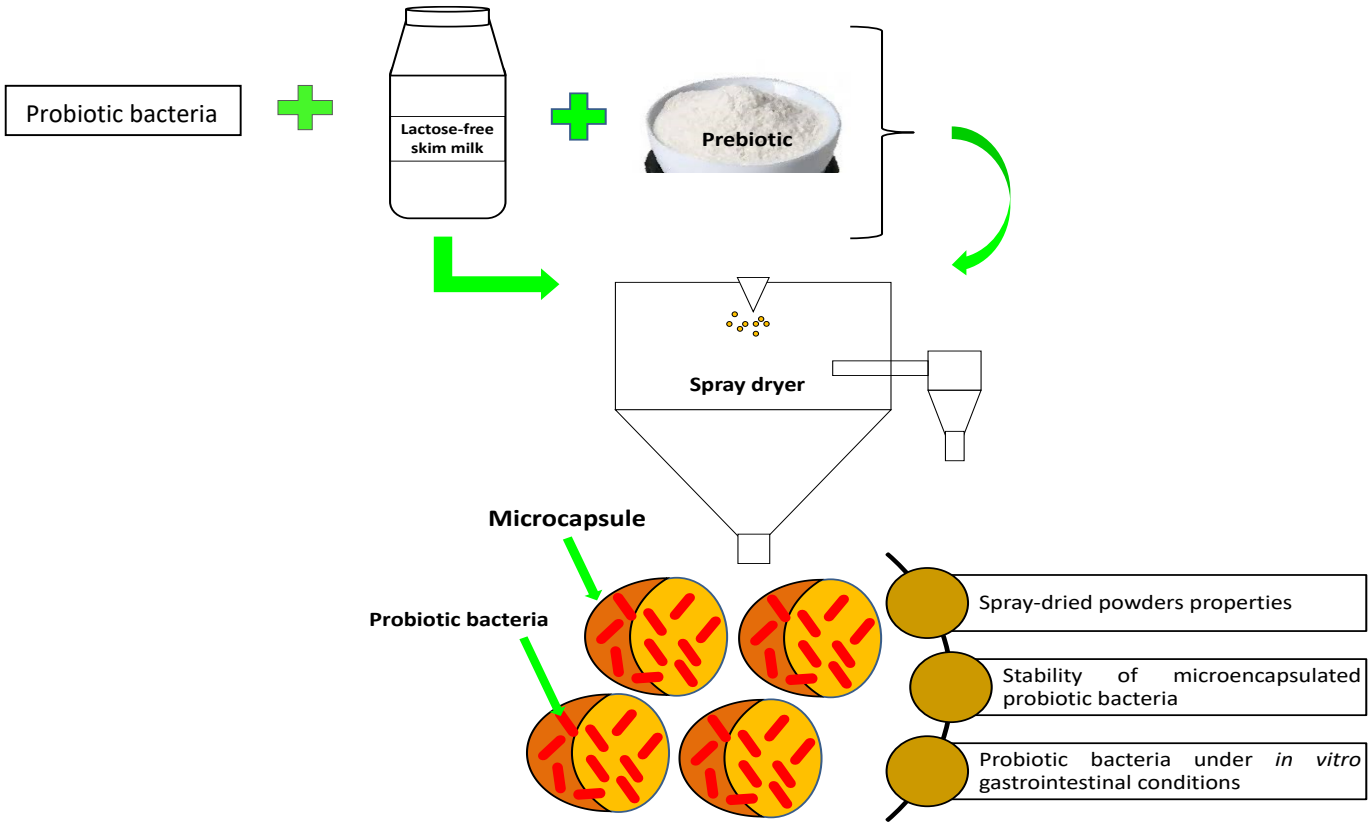
Lactose-free skim milk and prebiotics as carrier agents of *Bifidobacterium* BB-12 microencapsulation: physicochemical properties, survival during storage and *in vitro* gastrointestinal condition behavior

ABSTRACT

Bifidobacterium BB-12 was microencapsulated by spray drying using lactose-free milk, lactose-free milk and inulin, and lactose-free milk and oligofructose, resulting in powder 1, 2, and 3, respectively. The highest encapsulation yield (88.01%) and the highest bifidobacteria viability during 120 days of storage were noted for spray-dried powder 2. Spray-dried powders 1 and 3 showing a higher tendency to yellow color. After being submitted to in vitro simulated gastrointestinal conditions, the best probiotic survival rate result was found for spray-dried powder 3 (87.59%). Therefore, spray-dried powders containing prebiotics were the most appropriate combinations for microencapsulation of *Bifidobacterium* BB-12 and maintenance of cell viability during storage and gastrointestinal system, showing great potential to be used in lactose-free dairy products.

Keywords: Microencapsulation, free-lactose milk, inulin, oligofructose, bifidobacteria, gastrointestinal simulation



1 INTRODUCTION

Probiotic microorganisms are constantly studied because of their beneficial effects on human health, such as contribution to intestinal microbiota equilibrium and support for the immune system. Thus, the recommended minimum daily intake of probiotic viable cells is between 10^6 and 10^7 CFU per g of product (food matrix) (WANG *et al*., 2020). However, probiotic microorganisms experience unfavorable conditions when incorporated in food (for example, change in pH, temperature, and water activity). Furthermore, Mei *et al*. (2014) reported a considerable loss of probiotic cells viability as they pass through the low pH of the stomach and the high bile salt conditions of the intestine. Nevertheless, Verruck *et al*. (2018) stated that the stability of the probiotic cell can be obtained using the microencapsulation process by spray drying, through a combined utilization of carriers agents, also called wall materials. As for such agents, protein, polysaccharides, or a combination thereof are widely used. Among the polysaccharides, those with prebiotic properties, such as inulin and oligofructose, are commonly employed. These carbohydrates are not digested by the enzymes of the gastric tract (soluble fibre), and thus selectively stimulate the growth of beneficial bacteria in the colon (GIBSON *et al*., 2017). Both inulin and oligofructose are formed by a varying number of fructose moieties linked by β (2-1) glycosidic bonds. Our previous works shown that, after stress conditions (heat treatments, storage, and simulation of gastrointestinal digestion), the highest cell survivals were found for spray-dried powders that contained inulin and/or oligofructose as wall materials (FRITZEN-FREIRE *et al*., 2013; PINTO *et al*., 2015a; PINTO *et al*., 2015b; VERRUCK *et al*., 2017). Moreover, Verruck *et al*. (2018) reported a best thermal stability for probiotic microcapsules obtained from goat's milk and inulin. The measurement of such properties is extremely important, since heat treatments may be used in food processing. Apart from the works carried out by our group, the effectiveness of inulin in spray drying microencapsulation processes is already known and accepted by the scientific community. For example, Dos Santos *et al*. (2019) observed an improvement on *Lactobacillus acidophilus* La-5 survival after in vitro gastrointestinal stress when using inulin as carrier agent compared to the use of the free probiotic.

Microencapsulated probiotic bacteria have been incorporated into a wide range of foods, among them, milk and milk products have been successfully employed (GUL, 2017; PINTO *et al*., 2017; VERRUCK *et al*., 2020a). However, there is a significant segment of the adult world population (approximately 75%) showing permanent or temporary lactose intolerance (SURI *et al*. 2019). The prevalence of this inability to digest lactose may be

associated with the genetically programmed reduction in lactase activity during adulthood, which can cause severe digestive disorders (CORGNEAU *et al*., 2017). Given this world scenario of lactose intolerance, coupled with the fact that milk is an excellent carrier agent for probiotics (LEE *et al*., 2015), it is necessary to develop alternative mean that meets the demand formed by people who want to restrict lactose from their diet and consume probiotics. The microencapsulation of probiotics in a lactose-free milk matrix is an excellent choice since the addition of milk-based probiotics microcapsules in lactose-free dairy products constitutes a source of product contamination.

In this sense, our work seeks to evaluate the effect of lactose-free skim milk, oligofructose, and inulin as wall materials on the stability of *Bifidobacterium animalis* ssp. *lactis* BB-12, using the spray drying process. The stability of bifidobacteria entrapped was verified under in vitro simulated gastrointestinal conditions and during storage for 120 days at room temperature. The spray-dried powders were characterized concerning their physicochemical properties.

2 MATERIALS AND METHODS

2.1 PREPARATION OF PROBIOTIC CELLS

According to the procedure described by Fritzen-Freire *et al*. (2013), a stock solution was obtained from 25 g of *Bifidobacterium animalis* spp. *lactis* BB-12 freeze-dried culture (Nutrish® BB-12®, Chr. Hansen, Hønsholm, Denmark) rehydrated in 1 liter of sterile lactose-free milk with the following composition: 5.0 g 100 g^{-1} of carbohydrates, 3.2 g 100 g^{-1} of proteins and 0.40 g 100 g^{-1} of lipids. After the rehydration step, the stock solution was frozen at – 18 °C into sterile glass bottles. The stock solution was defrosted, inoculated in MRS broth (Difco, Sparks, USA) (150 mL L^{-1}), and incubated at 37 °C for 48 h, under anaerobic condition using anaerobic jars with AnaeroGen® (Oxoid, Hampshire, UK). In sequence, this cell suspension was centrifuged (Nova Técnica, São Paulo, Brazil) at 1,000 x *g* for 10 min at 25 °C. The supernatant was discarded, and the precipitate, which contained probiotics cells; was washed with a sterile saline solution (0.85 g 100 mL^{-1}) three times. Finally, it was obtained probiotics cells precipitate freshly prepared.

2.2 PREPARATION OF SPRAY-DRIED POWDERS WITH BIFIDOBACTERIA MICROCAPSULES

Three spray-dried powders with bifidobacteria microcapsules were obtained from three feed solutions with different compositions (Table 1). Lactose-free skim milk powder was used as basis for the three formulations. For this purpose, UHT lactose-free skim milk was purchased from the local market with the following composition: 5.0 g 100 g^{-1} of carbohydrates (2.5 g 100 g^{-1} of glucose and 2.5 g 100 g^{-1} of galactose), 3.3 g 100 g^{-1} of proteins, 0.0 g 100 g^{-1} of lipids and 0.21 g 100 g^{-1} of ash. This milk was subjected to a spray drying process (B 290 mini spray dryer, Buchi, Flawil, Switzerland), resulting in the lactose-free skim milk powder (85.5 g total solids 100 g^{-1}, 32.5 g protein 100 g^{-1}, 0.0 g lipid 100 g^{-1}, 3.0 g ash 100 g^{-1} and 50.0 g carbohydrates 100 g^{-1}). By direct correlation with its fluid version, we associated 50% of the total carbohydrates with galactose (25.0 g 100 g^{-1}) and 50% of the total carbohydrates with glucose (25.0 g 100 g^{-1}).

Table 1 − Feed solutions composition employed in *Bifidobacterium* BB-12 microencapsulation by spray drying.

Formulation	Carrier agent (g L^{-1})			Concentration of culture (mL L^{-1})
	Lactose-free skim milk powder	Inulin	Oligofructose	
Feed solution 1	200	-	-	100
Feed solution 2	100	100	-	100
Feed solution 3	100	-	100	100

Fonte: Os autores.

In the feed solution 1, only the lactose-free skim milk powder was used as a carrier agent, resulting in the spray-dried powder 1. For the feed solutions 2 and 3, inulin (DP ≥10) (92.10 g inulin 100 g^{-1} and 7.90 g fructose + glucose + sucrose 100 g^{-1}) (Orafti® Gr, Orafti, Tienen, Belgium) and oligofructose (DP = 2−8) (96.90 g oligofructose 100 g^{-1} and 3.10 g fructose + glucose + sucrose 100 g^{-1}) (Orafti® P95, Orafti, Tienen, Belgium) were incorporated, respectively. All feed solutions were homogenized, heat-treated at 80 °C for 30 min, and left to cool down to room temperature (25 °C). In sequence, probiotic cells precipitate freshly prepared were added in feed solutions at a concentration of 100 mL L^{-1}.

Microencapsulation processes were performed with a laboratory-scale spray dryer (B-290 mini spray dryer, Buchi, Flawil, Switzerland), operating at the constant air inlet temperature of 150 °C and the outlet temperature of 44 °C. For this, the feed solutions were kept under magnetic agitation (MS-3000, BioSan, Riga, Latvia) at room temperature and fed into the main chamber through a peristaltic pump, with feed flow of 12 mL min^{-1}, drying airflow rate of 35 m^3 h^{-1}, and compressor air pressure of 0.7 MPa. The spray-dried powders were collected from the cyclone base and placed under vacuum (200 B, Selovac, São Paulo, Brazil) in aluminum packaging. Three batches were produced and pooled for each one spray-dried powder type.

2.3 *BIFIDOBACTERIUM* BB-12 ENCAPSULATION YIELD

According to Sheu *et al*. (1993) but with some modifications, for the entrapped bacteria release of microcapsules, 1 g of spray-dried powder was previously re-suspended in 9 mL of sterile phosphate buffer solution (0.1 mol L^{-1}, pH = 7), and mixed in a vortex (VTX-F-100, Biomixer, São Paulo, Brazil) during 10 min, at room temperature (25 °C). Mixtures and feed solutions were serially diluted in peptone water (Oxoid, Hampshire, UK) (0.1 g 100 mL^{-1}), and plated on MRS agar (Merck, Darmstadt, Germany) modified with the addition of lithium chloride (Vetec, Rio de Janeiro, Brazil) (0.2 g 100 g^{-1}) and sodium propionate (Fluka, Neu-Ulm, Germany) (0.3 g 100 g^{-1}), as the methodology described by Vinderola and Reinheimer (1999). The plates were incubated in anaerobic jars containing AnaeroGen® at 37 °C for 72 h. After the incubation period, the count of viable probiotic cells was carried out and expressed as log colony-forming units per gram (log CFU g^{-1}). Therefore, the encapsulation yield was calculated as proposed by Chávarri *et al*. (2010) using the Eq. (1). All these determinations were realized in triplicate.

$$\text{Encapsulation yield (\%)} = \left(\frac{C}{C_0}\right) \times 100 \quad (1)$$

where C is the number of viable cells (log CFU) per gram in the spray-dried powders, and C_0 is the number of viable cells (log CFU) per gram in the feed solutions.

2.4 SPRAY-DRIED POWDER PROPERTIES

The lactose content and physical properties of all spray-dried powders were realized in triplicate.

2.4.1 Lactose content

Spray-dried powders lactose analysis was realized according to the methodology established by Steinbach and Wille (2008). Before analysis, spray-dried powders samples were diluted 1:100 (v/v) with ultrapure water and placed in the sample vials upon the rack of the sample processor. The subsequent dialysis of samples, followed by the injection of the dialysate onto the separation column of chromatography with pulsed amperometric detection (Chromatograph 881 Compact IC Pro, Metrohm AG, Herisau, Switzerland) was realized to determine the lactose content. Instrument control, data acquisition, and processing were performed by Metrodata IC Net software (Metrohm AG, Herisau, Switzerland). Lactose was reagent grade and purchased from (Sigma Aldrich, Buchs, Switzerland). It was employed columns Metrosep Carb 1 and Metrosep CO_3 Trap 1, and the pre-column Metrosep Carb 1 Guard (Metrohm AG, Herisau, Switzerland). The mobile phase used was a solution of sodium hydroxide (5.0 mmol L^{-1}), with a flow rate of 1.2 mL min^{-1} at a temperature of 45 °C.

2.4.2 Moisture, water activity and water solubility

The moisture content (g 100 g^{-1}) was determined by gravimetrically by oven drying under vacuum at 70 °C (Model TE-395, Tecnal®, Piracicaba, Brazil) until reaching constant weight, as described by AOAC (AOAC, 2005).

The water activity was measured in the Aqualab 4TE analyzer (Decagon Devices, Pullman, USA) at 25 °C, after the initial samples stabilization for 15 min, according to the method proposed by Fernandes *et al*. (2014), with some modifications. One gram of each spray-dried powder was weighed and stirred into 25 mL of distilled water for 5 min using a magnetic stirrer (HS-17, JoanLab®, Zhejiang, China) at a medium speed. The solution was then centrifuged (5430R, Eppendorf, Germany) at 760 x *g* for 10 min. An aliquot of 20 mL of the supernatant was transferred to a pre-weighed Petri dish and oven-dried at 105 °C overnight. The water solubility (%) was calculated as the percentage of dried supernatant about the amount of spray-dried powder originally added (1.0 g).

2.4.3 Bulk density and interstitial air

The density of spray-dried powders was measured both as loose and tapped bulk density, as proposed by Lebrun *et al*. (2012), but with some modifications. Approximately, 2 g of each spray-dried powder was freely poured into a glass tarred graduated cylinder without tapping and disturbance, and this was measured as loose bulk density of spray-dried powders, by the following equation:

$$\text{Loose bulk density} = \frac{\text{mass of spray-dried powder (g)}}{\text{bulk spray-dried powder volume } (cm^3)} \quad (2)$$

The spray-dried powders samples from loose bulk density evaluation were mechanically tapped, and after 100 taps were obtained the tapped bulk density, which was computed using the following equation:

$$\text{Tapped bulk density} = \frac{\text{mass of spray-dried powder (g)}}{\text{tapped spray-dried powder volume } (cm^3)} \quad (3)$$

Loose and tapped bulk densities values were used to determine the interstitial air value, as proposed by Chever *et al*. (2017), as follows:

$$\text{Interstitial air} = \left(\frac{1}{\text{Loose bulk density } (kg\ m^{-3})} - \frac{1}{\text{Tapped bulk density}(kg\ m^{-3})}\right) \text{X } 100000 \quad (4)$$

2.4.4 Flow properties

Carr's index (CI) and Hausner ratio (HR) were used to evaluating the flowability and cohesiveness of spray-dried powders, respectively. Both Carr's index and Hausner ratio were calculated according to the following equations given by Reddy *et al*. (2014):

$$\text{Carr's index (CI)} = \frac{\text{tapped bulk density} - \text{loose bulk density}}{\text{tapped bulk density}} \text{ X } 100\ (\%) \quad (5)$$

$$\text{Hausner ratio (HR)} = \frac{\text{tapped bulk density}}{\text{loose bulk density}} \quad (6)$$

2.4.5 Color measurements

Color measurements (L*, a*, and b* values) were performed using a chromameter CR-400 (Konica Minolta, Osaka, Japan) with illuminant D65. The instrument was calibrated with a white reference tile before the measurements. The L*, a* (+, red; - , green) and b* (+, yellow; - , blue) color coordinates were determined according to the CIELab coordinate color space system. Color measurements were performed on spray-dried powders at room temperature. The total color difference (ΔE*) was calculated as described by Himmetagaoglu and Erbay (2019), as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (7)$$

where ΔL* is the difference of luminosity, Δa* is the difference of the parameter a*, and Δb* represents the difference of the parameter b*, between two types of spray-dried powders.

2.5 EFFECT OF STORAGE ON MICROENCAPSULATED PROBIOTIC VIABILITY

Spray-dried powders (1, 2, and 3) with bifidobacteria microcapsules, placed under vacuum in aluminum packaging were stored at room temperature (25 °C) for 120 days. The samples were withdrawn at thirty days intervals to monitor the viable cells counts of *Bifidobacterium* BB-12, as previously described in item 2.3. These results were also expressed in log colony-forming units per gram (log CFU g^{-1}).

2.6 SURVIVAL OF BIFIDOBACTERIA UNDER IN VITRO SIMULATED GASTROINTESTINAL CONDITIONS ASSAY

Bifidobacterium BB-12 cells precipitate freshly prepared (free cells), and spray-dried powders (1, 2, and 3) were submitted to under *in vitro* simulated gastrointestinal conditions steps. The typical conditions prevailing in the human mouth, esophagus-stomach, duodenum, and ileum were sequentially simulated, as a traditional digestion step. Parameters (enzymes solutions, pH values, periods, and intensities of stirring in each part of the human digestive system) used to simulate the gastrointestinal conditions were realized exactly the protocol described by Verruck *et al*. (2017). As the control, all spray-dried powders with bifidobacteria microcapsules, and free cells samples were not also exposed to the simulated gastrointestinal

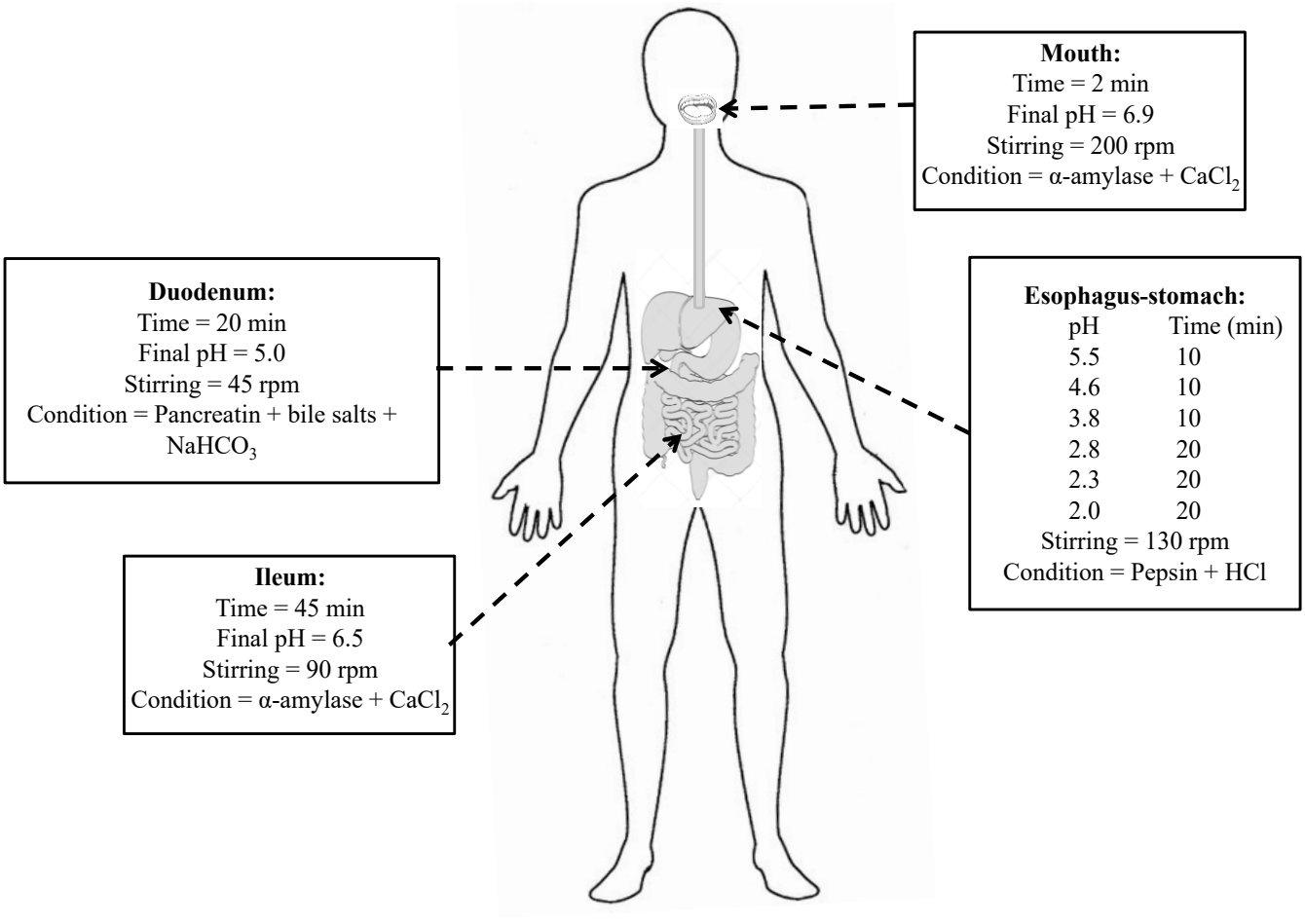
conditions. The processing conditions used in each step of the simulated gastrointestinal are summarized in Figure 1. After each step condition, viable cell counts of *Bifidobacterium* BB-12 were done as also previously described in item 2.3. This assay was carried out in triplicate, and results were exhibited as log colony-forming units per gram (log CFU g^{-1}).

The survival (%) of bifidobacteria was calculated as proposed by Guo *et al*. (2009) using the equation (12):

$$\text{Survival (\%)} = \left(\frac{N}{N_0}\right) \text{x } 100 \quad (8)$$

where N is the viable cell count of bifidobacteria (it expressed as log colony-forming units per gram [log CFU g^{-1}]) after exposure to each step of simulated gastrointestinal conditions, and N_0 is the initial viable cell count of bifidobacteria (it expressed as log colony-forming units per gram [log CFU g^{-1}]) before to simulated gastrointestinal conditions.

Figure 1 − Protocol of under in vitro simulated gastrointestinal conditions steps according to Verruck *et al*. (2017). All enzymes and bovine bile salts were purchased from Sigma Aldrich (St. Louis, USA), while all others reagents were of analytical grade.



Fonte: Os autores.

2.7 STATISTICAL ANALYSIS

To determine significant differences ($P < 0.05$) between results, one-way analysis of variance (ANOVA) and Tukey's studentised range test were used. All statistical analyses were performed using STATISTICA 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). All data were expressed as mean ± standard deviation.

3 RESULTS AND DISCUSSION

3.1 *BIFIDOBACTERIUM* BB-12 ENCAPSULATION YIELD

The viable cell count for the bifidobacteria in the feed solutions and their respective spray-dried powders are shown in Table 2. All the spray-dried powders showed viable cell counts above 6 log CFU g^{-1}, which is the minimum recommended amount for the probiotic product claim. Furthermore, there were no significant differences ($P > 0.05$) between the powders for cell survival after spray drying. Similar behavior was observed by Verruck *et al*. (2019) and by Pinto *et al*. (2015a) for microcapsules produced with reconstituted full-fat goat's milk powder/prebiotic agents and whey concentrate/prebiotic agents, respectively. The highest values of encapsulation yield (EY) were noted for the spray-dried powders produced with inulin and oligofructose, consecutively. For these same carrier agents, some authors (FRITZEN-FREIRE *et al*., 2012; KINGWATEE *et al*., 2015; RAJAM; ANANDHARAMAKRISHNAN, 2015) had already reported a thermoprotective effect on probiotic microorganisms during spray drying processes. On the other hand, according to Ananta *et al*. (2005), large chains polymers would not interact directly with the polar head groups of cell membrane phospholipids, reducing its protection during the drying process. Besides, the dehydration involved in the spray drying process generally results in injury or death to bifidobacteria cells (VERRUCK *et al*., 2020b). However, our results were satisfactory, and according to Dias *et al*. (2018), this may be attributed to the low outlet temperature kept in the spray dryer equipment (44 ± 3 °C), in addition to the natural resistance of the microorganism, because as verified in the work of Verruck *et al*. (2017), the counts of free *Bifidobacterium* BB-12 remained above 6 log UFC g^{-1} when they were exposed to a temperature of 55 ºC for 15 min. Moreover, Verruck *et al*. (2019) affirm that due to the presence of milk proteins and lactose, dairy products are effective in cell protection during spray drying processes. During water removal, these compounds would prevent the membrane from

breaking through their interaction with it. As there is no lactose in our powders, we associate the good viability, especially with proteins. The findings of Wang *et al*. (2020) corroborate with this since these authors found a more significant role of milk proteins than lactose in bacterium protection during dehydration. The authors also verified higher respiratory activity and membrane integrity of bacteria for treatments with more proteins. Furthermore, according to Ying *et al*. (2013), the bovine whey protein creates a buffered environment within the particle obtained by spray drying.

We also highlight that our samples contain the monosaccharides glucose and galactose, given the previous enzymatic hydrolysis of lactose. In this context, Amaretti *et al*. (2007) studied kinetics and metabolism of *Bifidobacterium adolescentis* growing, and they found a greater growth rate and cellular yield when they were subjected to growth on galactose than glucose, lactose, and galactooligosaccharides. According to Prasanna *et al*. (2014), although some strains can grow in milk and utilize lactose as the substrate, the genus *Bifidobacterium* includes saccharolytic organisms and is characterized mainly by to ferment glucose, galactose, and fructose.

Table 2 − Viable *Bifidobacterium* BB-12 cells count and encapsulation yields (EY)

Samples	Viable cells (log CFU g^{-1})	EY (%)
Feed solution 1	10.65 ± 0.04^{a}	86.66
Spray-dried powder 1	9.23 ± 0.05^{b}	
Feed solution 2	10.55 ± 0.13^{a}	88.01
Spray-dried powder 2	9.29 ± 0.07^{b}	
Feed solution 3	10.62 ± 0.02^{a}	87.52
Spray-dried powder 3	9.30 ± 0.04^{b}	

Feed solution 1 and Spray-dried powder 1 are the *Bifidobacterium* solution with addition of lactose-free skim milk powder, and its spray-dried powder, respectively. Feed solution 2 and Spray-dried powder 2 are the *Bifidobacterium* solution with addition of lactose-free skim milk powder and inulin, and its spray-dried powder, respectively. Feed solution 3 and Spray-dried powder 3 are the *Bifidobacterium* solution with addition of lactose-free skim milk powder and oligofructose, and its spray-dried powder, respectively. [a-b]Within a column, means ± standard deviations with different superscript lowercase letters denote significant differences ($P < 0.05$) among the samples.

3.2 SPRAY-DRIED POWDERS PROPERTIES

The physicochemical characteristics of the spray-dried powders are shown in Table 3. The presence of lactose was not detected in any of the spray-dried powders by the method used in this work, thus ensuring that this product can be called lactose free. The moisture content of the spray-dried powders 2 and 3 were similar, as well as their water activity. In turn, the moisture content and water activity values of the spray-dried powder 1 were highest. It is known that glucose and galactose present in milk are highly reactive when compared to disaccharide lactose (MILKOVSKA-STAMENOVA; HOFFMANN, 2016). Besides, both glucose and galactose have lower glass transition temperature (T_g) than inulin and oligofructose (31, 30, 132, and 102 ºC, respectively) (SCHUCKck *et al*., 2005; SILVA *et al*., 2016; HINRICHS *et al*., 2001). In our case, the spray-dried powder 1 certainly had lower T_g than the other two samples, due to the T_g theory resulting from the composition of the product proposed by Couchman and Karasz (1978). A glass transition temperature close to room temperature facilitates water absorption (JULIANO; BARBOSA-CÁNOVAS, 2010) and as a result, it can bring a series of problems to milk powder such as particle agglomeration, cacking, and rehydration difficulty. Also, the lower the degree of polymerization of a component, the more hygroscopic it will be (JIMENEZ-SÁNCHEZ *et al*., 2018), which also justifies the higher moisture value found for powder 1 since glucose and galactose are simpler carbohydrates compared to the prebiotics used in the other formulations. However, despite this higher value found for spray-dried powder 1, the results are still within the recommended range, because according to Riveros *et al*. (2009), the spray dryer operating conditions should be set to reach temperatures close to or below 60 °C at the air discharge point to assure the obtainment of a product with less than 10 g 100 g^{-1} moisture. These results are like to that described by Rajam and Anandharamakrishnan (2015); they found a moisture content between 5.52 and 7.43 g 100 g^{-1} in the powders obtained in microencapsulation by spray drying of *L. plantarum* using as carriers agents fructooligosaccharide and whey protein isolate, and fructooligosaccharide and denatured whey protein isolate, with an outlet temperature of 55 °C. Ilha *et al*. (2015) report moisture content 4.30 g 100 g^{-1} for *L. paracasei* spray dried in reconstituted skim milk and cheese whey. Wang *et al*. (2020) suggested that ideal water activity for probiotic stability must be less than or equal to 0.4. Our water activity results were similar to those found by Fritzen-Freire *et al*. (2012), who microencapsulated *Bifidobacterium* BB-12 in reconstituted skim milk, inulin, and oligofructose and obtained water activity values between 0.21 and 0.27.

Table 3 − Physicochemical properties of probiotic spray-dried powders obtained from lactose free skim milk and prebiotics

	Spray-dried powders		
	1	2	3
Lactose (%)	nd	nd	nd
Moisture (g 100 g^{-1})	7.67 ± 0.12^{a}	4.45 ± 0.28^{b}	4.54 ± 0.09^{b}
Water activity	0.396 ± 0.004^{a}	0.276 ± 0.006^{b}	0.288 ± 0.009^{b}
Solubility (%)	63.10 ± 0.51^{a}	62.39 ± 1.22^{a}	62.75 ± 1.52^{a}
Loose bulk density (g cm^{-3})	0.32 ± 0.02^{b}	0.32 ± 0.01^{b}	0.44 ± 0.05^{a}
Tapped bulk density (g cm^{-3})	0.37 ± 0.03^{b}	0.42 ± 0.02^{b}	0.53 ± 0.06^{a}
Interstitial air (IA) (cm^{-3}100 g)	42.21 ± 4.54^{b}	61.18 ± 2.36^{a}	42.61 ± 1.91^{b}
Flowability (Carr's index) (%)	12.68 ± 2.58^{b}	23.43 ± 5.99^{a}	17.65 ± 0.36^{ab}
Cohesiveness (Hausner ratio)	1.15 ± 0.03^{b}	1.31 ± 0.11^{a}	1.21 ± 0.01^{ab}
*L**	95.58 ± 0.32^{a}	93.90 ± 1.81^{a}	93.93 ± 0.35^{a}
*a**	0.26 ± 0.04^{b}	0.04 ± 0.01^{c}	0.37 ± 0.06^{a}
*b**	8.87 ± 0.42^{a}	6.68 ± 0.06^{b}	9.37 ± 0.04^{a}
h	88.31 ± 0.19^{b}	89.69 ± 0.05^{a}	87.74 ± 0.36^{b}
ΔE*	-	2.76	1.72

[a–c]Within a line, means ± standard deviations with different superscript lowercase letters denote significant differences ($P < 0.05$) between the samples.
nd: not detected, (1): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free skim milk powder, (2): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and inulin, (3): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and oligofructose.

The solubility is a useful parameter for the application of the powders in various matrices, which is dependent on the affinity of the powders to water and hydrophilic components (Rodriguéz-Restrepo *et al*., 2017). Sadat *et al*. (2017) affirmed that the main factors affecting the solubility of milk powders are drying conditions and the physical characteristics of the feed liquid (e.g., viscosity). According to Himmetagaoglu and Erbay (2019), the composition of the powder also provides a considerable effect on this parameter. However, the solubility values found in our study did not vary significantly as a function of carrier agents used, remaining on average 63%. Kalita *et al*. (2018) also obtained solubility values in the range of 62 to 68% for symbiotic spray-dried powders from maltodextrin and fructooligosaccharide. Low solubility rates result in a slower release of microencapsulated cells since the rehydration of the powders also occurs more slowly (PINTO *et al*. 2015a). Therefore, the lowest solubility

rates contribute to the longest microcapsule dissolution time. Pinto *et al*. (2015b) highlighted that by adding microcapsules in food products, it is expected that there is good control of the release of the probiotic cells when in contact with an aqueous solution, and a longer dissolution time assists in this regard.

The bulk density can be affected by some parameters such as moisture content, particle size distribution, and morphology and it is important for processing, storage, and packaging of powders (RAJAM; ANANDHARAMAKRISHNAN, 2015). It was possible to note, both for loose bulk density and for tapped bulk density, that the use of oligofructose as the carrier agent contributed to the lowest volume occupied of the spray-dried powder 3 (Table 3). Rajam and Anandharamakrishnan (2015) also found higher loose bulk density values for microcapsules with a higher content of fructooligosaccharide. They attributed this behavior to the fact that microcapsules with higher fructooligosaccharide ratio presented particle aggregation and less interspace between particles. Furthermore, our values are similar to those found in other studies. For example, De Liz *et al*. (2020) found variations between 0.32–0.33 g cm^{-3}, and 0.54–0.55 g cm^{-3}, respectively for loose and tapped bulk densities of spray-dried powders with goat's whey freeze concentrate and inulin, and with only goat's whey freeze concentrate, respectively. Looi *et al*. (2019) found 0.36–0.45 g cm^{-3} and 0.52–0.64 g cm^{-3}, respectively for loose and tapped bulk densities for probiotic spray-dried powders from *Moringa oleifera* Lam. The change in specific volume between the loose and tapped bulks is recognized as interstitial air content (IA) (WU *et al*., 2019). The IA was highest ($P < 0.05$) for the spray-dried powder 2 (with the addition of inulin), and the values were lower and without statistical differences ($P > 0.05$) for both spray-dried powders 1 and 3. As observed in the work of Chever *et al*. (2017), IA values tend to decrease proportionally with the increase of the agglomeration of a powder.

There were variations in the values of flowability and cohesiveness between the three spray-dried powders. According to Parthasarathi and Anandharamakrishnan (2016), these flow properties are important quality parameters for the industrial production of dried microcapsules. These same authors state that Carr's index (CI) results above 38% are classified as "very, very poor" flowability. They also state that a Hausner ratio greater than or equal to 1.25 indicates a powder with "poor" flow characteristics. Our spray-dried powders showed a flowability < 30% for all samples, and Hausner ratio < 1.25 for powders 1 and 3, and > 1.25 for powder 2. Thus, according to the tables of classification of the flowability and cohesiveness of powders arranged in the work of Jinapong *et al*. (2008), our results reveal very good flowability and low cohesiveness for powder 1, fair flowability and intermediate cohesiveness for powder 2, and good flowability and intermediate cohesiveness for powder 3. In the studies of Fitzpatrick *et al*.

(2004), it is evidenced that the skim milk powder has low cohesiveness when compared to its whole version because of its zero fat content. One of the mechanisms of the formation of particle agglomerates is through solid bridges. These can be built up by chemical reaction, crystallization of dissolved binder substances, hardening binders, solidification of melted components, and by sintering at high temperatures (through the diffusion of molecules from one particle to another at the points of contact). Highly viscous materials and high molecular weight organic liquids can form bonds very similar to those of solid bridges (PIETSCH, 1997). We suggest, therefore, that the prebiotics used in our study were responsible for the drop in quality in the flow properties.

The colour parameters for the *Bifidobacterium* BB-12 spray-dried powders samples also are shown in Table 3. All the spray-dried powders showed high values for the L^* parameter, indicating that the samples were white (clearer). Concerning the a^* and b^* parameters, the spray-dried powders showed positive values, indicating a tendency to the colour red and the colour yellow, respectively. The use of lactose-free milk or lactose-free milk and oligofructose produced spray-dried powders with a more yellowish coloration. We believe that the high contents of proteins and reducing sugars (mainly galactose and glucose) in spray-dried powder 1 favored Maillard reactions during thermal processing. Mensink *et al*. (2015) reported that the hydrolysis of inulin into oligofructose results in a product with high reducing capacity, and therefore, is susceptible to the Maillard reaction. Thus, it was found that in spray-dried powders 1 and 3 there were more reducing sugars, resulting in a more pronounced yellowish color when compared to spray-dried powder 2.

The values of Hue angle (h) showed that the spray-dried powder 2 differed significantly from the others, suggesting that this change does not go unnoticed to human eyes. According to Dobrzańska and Cais-Sokolińska (2014), who studied the color measurement systems focused on the protein of milk and whey, a total color difference (ΔE^*) > 2 would already be perceived by an inexperienced observer. Complementing the values found for the Hue angle, this was also observed for the spray-dried powder 2. In the study of Witczak *et al*. (2020), a higher ΔE^* was found for samples containing inulin with a higher degree of polymerization. However, the authors state that the color differences found are not only correlated with DP but also due to the interaction between inulin and other system components, as well as the technologies utilized in the production of this prebiotic. In agreement with the data obtained by Witczak *et al*. (2020), our results suggest that the greater degree of polymerization of inulin contributed to the different values of h and ΔE^* for spray-dried powder 2, results that originated from the differences observed ($P < 0.05$) in a^* and b^* parameters.

3.3 EFFECT OF STORAGE ON MICROENCAPSULATED PROBIOTIC VIABILITY

The viable *Bifidobacterium* BB-12 cell counts throughout the storage time of 120 days at 25 ± 1°C are shown in Table 4. During storage intervals, the spray-dried powders containing microencapsulated bifidobacteria showed a decrease in viable cell count. The protection performance was lower when only lactose-free milk powder was used as the carrier agent, reaching a loss of almost 100% of its viability. Gul (2017) discussed that the viability of microencapsulated cells during storage depends on various factors such as a high number of irreversible damage cells during spray drying, presence of oxygen, high storage temperature, moisture content, product composition, and exposure to light. According to Liu *et al*. (2016), the maintenance of the moisture content of microcapsules around 4 g 100 g^{-1} is important because as a higher the moisture content, lower bacterial survival during storage. Thus, the remarkable decrease in viability of the spray-dried powder 1 could be associated with its initial moisture and a_w, which as shown in Table 3, were 7.67 g 100 g^{-1} and 0.396, respectively. Moreover, the decrease in the viable cell count during storage at 25° C may be correlated with the natural mechanism that involves the degradation of life-essential macromolecules. For example, Santivarangkna, Kulozik and Foerst (2008) affirmed that this viability loss is mainly due to membrane lipid oxidation. The cell counts remained higher than 6 log CFU g^{-1} for spray-dried powder 2 (made with inulin and lactose-free skim milk as carrier agents) for 90 days, while for spray-dried powders 1 and 3 (made with the lactose-free skim milk powder, and lactose-free skim milk powder and oligofructose, respectively) they remained higher than 6 log CFU g^{-1} only for 30 days. This result is following those obtained by Dias *et al*. (2018), who noted that microcapsules with a higher proportion of inulin increased cell viability of *B. animalis* ssp. *lactis* BB-12 compared with samples with no addition of inulin or with a low concentration of this prebiotic, during storage at 25° C. As discussed in the previous item, the use of different carrier agents resulted in spray-dried powders with different physical properties, and according to Verruck *et al*. (2019), this affects the functionality of the microcapsules present. Bedani, Rossi and Saad (2013) cited that the inulin interacts with available water, forming a gel that consists of a tridimensional network of microcrystals. This structure may involve the bacterial cells, contributing to physical protection and consequent maintenance of viability. Moreover, this fact may be associated to the elevated glass transition temperature inulin presents. It was reported that a carbohydrate-rich formulation with high T_g procured the greater stability for probiotic bacteria (ROKKA; RANTAMAKI, 2010; NUNES *et al*., 2018). Zhang, Lin and Zhong (2015) observed that the use of trehalose in feed solutions provided

better survivability of *Lactobacillus salivarus* NRRL B-30514 (after spray drying) during storage than powders made with only reconstituted skimmed milk. Muhammad *et al*. (2017) explained that when the carrier agent is at the glassy state (below T_g), its viscosity is higher, which decelerates chemical reactions such as free radical oxidation. Thus, the glassy state could prevent further cellular destruction in spray-dried powders, henceforth supplying additional safeguard to the cells. In addition, the positive effect of inulin may be attributed to its prebiotic properties, since this carbohydrate is selective consumed by bifidobacteria.

Table 4 − Viable *Bifidobacterium* BB-12 cells count from spray-dried powders during 120 days of storage at 25 ± 1 °C

	Viable cell count of the spray-dried powders (log CFU g^{-1})		
Storage (days)	1	2	3
0	9.23 ± 0.05^{a}	9.29 ± 0.07^{a}	9.30 ± 0.04^{a}
30	8.18 ± 0.16^{b}	9.24 ± 0.01^{a}	9.13 ± 0.08^{a}
60	1.95 ± 0.07^{c}	7.81 ± 0.02^{b}	5.88 ± 0.13^{b}
90	1.78 ± 0.25^{c}	6.50 ± 0.43^{c}	4.08 ± 0.18^{c}
120	0.17 ± 0.01^{d}	4.49 ± 0.50^{d}	2.23 ± 0.23^{d}

1 is the spray-dried powder that was prepared employing the Feed solution 1, with 200 g of lactose-free skim milk powder. 2 is the spray-dried powder that was prepared using the Feed solution 2, with 100 g of inulin and 100 g of lactose-free skim milk powder. 3 is the spray-dried powder prepared using the Feed solution 3, with 100 g of oligofructose and 100 g of lactose-free skim milk powder. $^{a-d}$Within a column and for the same temperature, different superscript lowercase letters denote significant differences ($P < 0.05$) among the samples at different storage day.

3.4 SURVIVAL OF BIFIDOBACTERIA UNDER *IN VITRO*-SIMULATED GASTROINTESTINAL CONDITIONS ASSAY

The main objective of this experiment was to evaluate the viability of *Bifidobacterium* BB-12 during their transition through the mouth to the small intestine. The Table 5 and Fig. 2 show, respectively, the survival (%) and the viable cell counts of the free and of the microencapsulated *Bifidobacterium* BB-12 exposed (1, 2, 3 and free cells) and of those not exposed (1C, 2C, 3C and free cells C, i. e., controls) to the *in vitro* simulated gastrointestinal conditions. As the results of the control samples did not change during the assay, it is clear that external factors did not act on the bacteria, and thus, the effectiveness of the *in vitro* simulated gastrointestinal conditions was proven.

The first step was the simulation of mouth conditions. After this step, the free cells showed a decrease ($P < 0.05$) in viable *Bifidobacterium* BB-12 cell count; whereas no differences ($P > 0.05$) in the viable cell count of all the spray-dried powders (1, 2, and 3) were detected after exposure to the same condition. Thus, it was possible to note that the microencapsulation process protected the bifidobacteria in this step. Similar behavior was observed by Verruck *et al*. (2017), who microencapsulated *Bifidobacterium* BB-12 in different matrices, and verified a decrease in the viability of free cells when submitted to simulated mouth conditions. These authors also reported that the maintenance of cell viability in the spray-dried powders after this step is related to the factors such as the buffering capacity of the $NaHCO_3$ solution (used for pH adjustment), and the smaller contact surface between α-amylase and the bifidobacteria.

Table 5 − Survival (%) of *Bifidobacterium* BB-12 free and microencapsulated after each step of the under simulated gastrointestinal conditions

Spray-dried powder	Mouth	Esophagus-Stomach	Duodenum	Ileum
1	100.68 ± 1.02^{aA}	78.07 ± 5.34^{bcB}	70.52 ± 1.82^{cC}	80.01 ± 1.08^{bC}
1C	100.85 ± 0.82^{aA}	99.75 ± 0.55^{aA}	99.58 ± 0.39^{aA}	99.50 ± 0.91^{aA}
2	100.40 ± 0.16^{aA}	80.80 ± 1.61^{bB}	78.19 ± 1.80^{bB}	81.29 ± 0.76^{bC}
2C	99.44 ± 1.95^{aA}	97.72 ± 0.48^{aA}	97.75 ± 0.44^{aA}	98.74 ± 0.64^{aA}
3	100.25 ± 0.31^{aA}	83.72 ± 0.42^{bB}	67.36 ± 2.95^{cC}	87.59 ± 0.02^{bB}
3C	100.04 ± 0.72^{aA}	100.39 ± 3.36^{aA}	99.28 ± 1.80^{aA}	101.66 ± 1.57^{aA}
Free cells	90.10 ± 0.37^{aB}	81.34 ± 6.97^{abB}	44.68 ± 3.73^{cD}	75.53 ± 0.85^{bD}
Free cells C	100.15 ± 0.19^{aA}	99.10 ± 0.46^{aA}	100.41 ± 0.18^{aA}	99.35 ± 1.32^{aA}

[a–c]Within a line, means ± standard deviations with different superscript lowercase letters denote significant differences ($P < 0.05$) among different steps of the simulated gastrointestinal conditions for each sample.
[A–D]Within a column, means ± standard deviation with different superscript uppercase letters in the same column indicate significant differences ($P < 0.05$) among the same step of the simulated gastrointestinal conditions for all samples.
(1): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free skim milk powder, (2): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and inulin, (3): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and oligofructose. The letter C after each respective identification represent the samples not exposed to the simulated gastrointestinal conditions, i.e., used only as control.

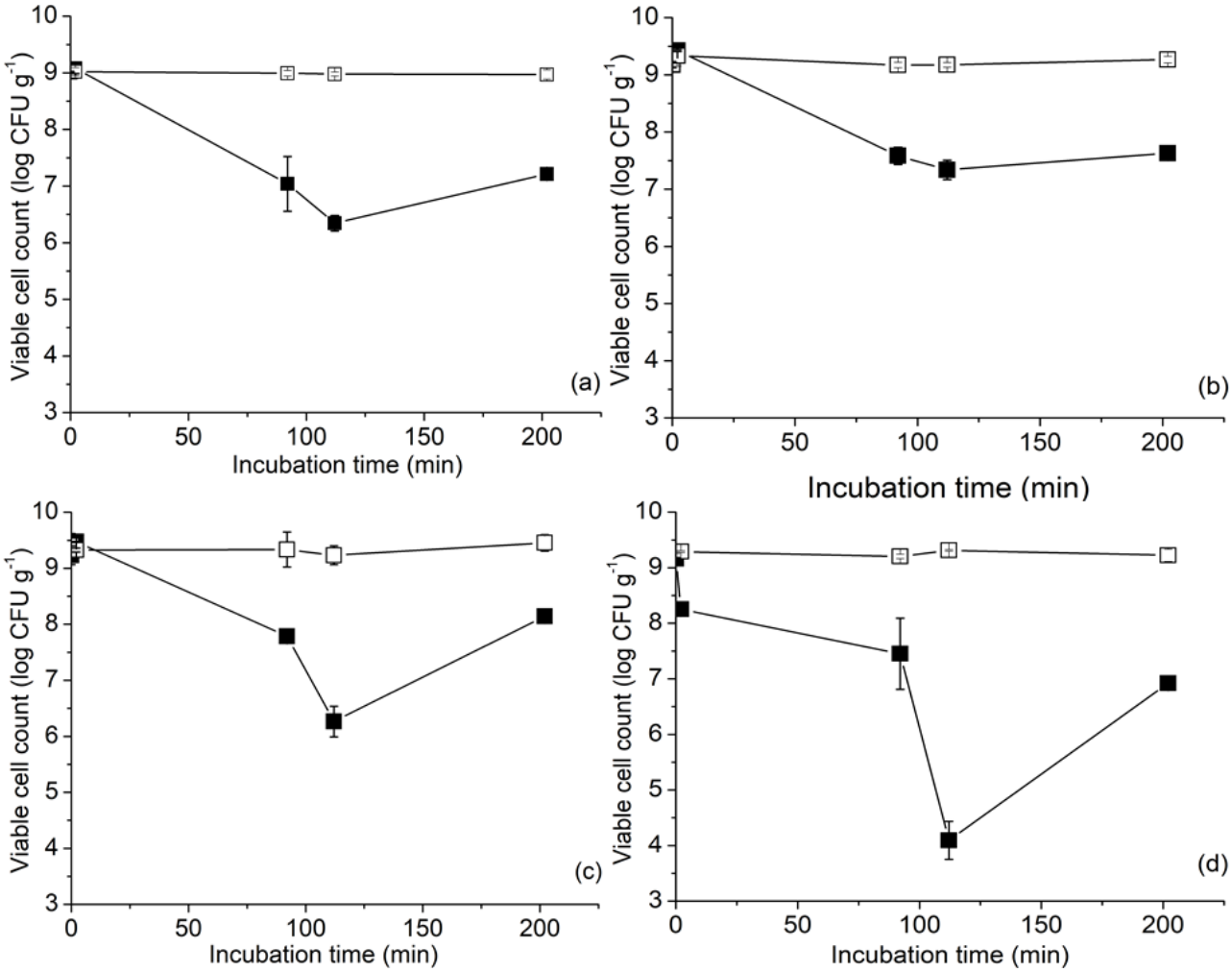
After exposure to the simulated esophagus-stomach conditions, both the viable cell counts and the survival (%) of *Bifidobacterium* BB-12 decreased ($P < 0.05$) for all samples. When analyzing this step separately, it was verified that there were no significant differences between all spray-dried powders and free cells, which suggests sensitivity towards simulated

gastric juice containing HCl and pepsin. Liu *et al*. (2016) noticed that the dissolution of many microcapsules and a loss of protection to the bacteria is strongly correlated to low pH.

The highest protective effect of the spray-dried powders on probiotic survival was observed in the duodenum step of the *in vitro* simulated gastrointestinal conditions. The free cells bacterial viability experienced a dramatic decrease in the duodenal phase when compared to the microencapsulated forms. As stated by Madureira *et al*. (2011), the amphiphilic nature of bile salts enables its strong inhibitory effect on bacteria, thus heavily constraining bacterial survival throughout the gastrointestinal tract. Thereby, the free cells were apparently in a more fragile state when they reached this step. This occurs due to damages caused by the acidic conditions before exposure to the duodenum conditions, thus recovery of probiotic cells was eventually not possible. Ranadheera *et al*. (2014) also found similar behavior when submitted unencapsulated probiotics to the presence of bile salts during in vitro gastrointestinal tolerance, that is, the cells experienced a significant decrease in their viability. On the other hand, the increase of pH in the duodenum step appeared to be favorable for the survival of probiotic cells in the spray-dried powder 2, since it exhibited higher stability than the other samples. This behavior suggests that lactose-free skim milk in association with inulin may lower toxicity of bile on membrane damage; similar results were reported by Kingwatee *et al*. (2015) and Fritzen-Freire *et al*. (2013).

Remarkably, from the duodenum step to the ileum step, the results of the viable cell count and the survival for the spray-dried powder 2 showed no differences ($P > 0.05$). For the spray-dried powders 1 and 3, as well as for the free cells these parameters increased ($P < 0.05$). Verruck *et al*. (2017) observed the same phenomenon in a microcapsule produced with goat's milk, inulin, and oligofructose, and for free bifidobacteria. Verruck *et al*. (2015) reported that this increase cannot reasonably be attributed to cell multiplication, and probably resulted from a massive release of uninjured bifidobacteria from degraded microcapsules and/or recovery of sublethally injured cells. Besides, Moumita *et al*. (2017) observed that due to acidity shock in the stomach, some of the lineages of lactobacilli entered a dormant state, and regain their growth when the pH in the small intestine reached 6.0. Therefore, at the end of the simulated gastrointestinal conditions, *Bifidobacterium* BB-12 contained in spray-dried powder 3 showed the highest ($P < 0.05$) survival, followed by the spray-dried powders 1 and 2, which did not show differences between them ($P > 0.05$). Free cells exhibited the lowest viability value.

Figure 2 − Survival of free and microencapsulated *Bifidobacterium* BB-12 after each step of the simulated gastrointestinal conditions.



(a) 1 (■) and 1C (□); (b) 2 (■) and 2C (□); (c) 3 (■) and 3C (□); (d) Free cells (■) and free cells C (□). (1): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free skim milk powder, (2): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free milk powder and inulin, (3): spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free skim milk powder and oligofructose. The letter C after each respective identification represent the samples not exposed to the simulated gastrointestinal conditions, i.e., used only as control. The error bars represent standard deviation of mean.

4 CONCLUSIONS

While investigating the effect of lactose-free skim milk powder and prebiotics in spray drying of *Bifidobacterium* BB-12, we observed that, all the formulations showed overall positive effects. However, microparticles made from lactose-free skim milk powder and inulin gave better result for survivability of probiotic bacteria during storage. The spray-dried powder produced with lactose-free skim milk powder and oligofructose showed the highest probiotic survival at the end of simulated gastrointestinal conditions. On this approach, we concluded

that the spray-dried powders containing prebiotics (2 and 3) were the most appropriate combinations for microencapsulation of *Bifidobacterium* BB-12 and maintenance of cell viability during storage and gastrointestinal system, showing great potential to be used in lactose-free dairy products.

ACKNOWLEDGMENTS

The authors are grateful to Coordination of Improvement of Higher Education Personnel (CAPES, Brazil) [CAPES-PRINT, Project number 88887.310560/2018-00]; to National Council for Scientific and Technological Development (CNPq, Brazil) by the financial support [CNPq, 405965/2016-8], and to Coordination of Improvement of Higher Education Personnel (CAPES, Brazil) by the scholarship [001].

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CAPÍTULO 3

Current knowledge about physical properties of innovative probiotic spray-dried powders produced with lactose-free milk and prebiotics

*** Trabalho publicado (redação na íntegra)**

LWT - Food Science and Technology 151 (2021) 112175

Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/lwt

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ARTICLE INFO

ABSTRACT

Current knowledge about physical properties of innovative probiotic spray-dried powders produced with lactose-free milk and prebiotics

ABSTRACT

Lactose-free probiotic powders were obtained by mixing *Bifidobacterium* BB-12 suspensions with lactose-free milk powder or lactose-free milk powder and prebiotics (inulin or oligofructose). A thorough investigation was performed to know their water sorption properties and physical and thermal characteristics. By evaluating the water sorption properties, the Peleg model fitted well to the experimental sorption data, showing that the equilibrium moisture content of powders increased as the relative humidity increased. The isotherm found for all samples was a Type III Isotherm, commonly observed in most foods. For both morphology and particle size, the use of different carrier agents affected these properties; however, all the spray-dried powders presented good size to be added in food products. X-ray diffraction and Raman spectroscopy showed us amorphous structure for all powders, and typical bands of the milk constituents and sugars, respectively. By evaluating the spray-dried powders thermal properties, it was possible to confirm that the addition of prebiotics gave higher thermal stability, highlighting the sample produced with inulin. We concluded that a good quality of lactose-free milk based probiotic powder could be obtained using spray drying technique, with great potential to be applied in lactose-free dairy products.

Keywords: *Bifidobacterium* BB-12, microencapsulation, lactose-free microspheres, physical characterization, prebiotics.

1 INTRODUCTION

The probiotics market was valued at USD 48.88 billion in 2019 and is estimated to increase to USD 94.48 billion by 2027. This expansion is owing to consumer perception about the health benefits of probiotic-based products, in addition to the demand for immunity-boosting products amid COVID-19 (MARKET RESEARCH REPORT, 2020). In this context, the microencapsulation of probiotic cells within dairy matrices is already a widespread and accepted technology, as well as the joint use of prebiotic carbohydrates. Due to the unfavorable conditions encountered by probiotic microorganisms when present in a food matrix (for example, changes in pH, water activity, temperature, and oxygen content), the production of powdered probiotic ingredients is necessary. In addition to ensuring the arrival to the colon in adequate quantities (VERRUCK *et al*., 2017), this type of presentation allows longer storage and versatility in applications.

Milk-based probiotic powders have already ready been applied in several types of dairy products (PINTO *et al*., 2017; VERRUCK *et al*., 2020; GUL, 2017), including lactose-free dairy (PINTO *et al*., 2019). However, the addition of these microspheres in lactose-free dairy constitutes a source of product contamination. Given the world scenario of lactose intolerance (about 70% of the world's adult population is lactose-intolerant) (LULE *et al*., 2016), products free of this disaccharide are essential to make up the diet of intolerant people. In this state, the individual cannot digest and absorb dietary lactose, due to deficiency in β-galactosidase, the enzyme that is responsible for lactose hydrolysis. This condition leads to gastrointestinal manifestations such as diarrhea, vomiting, abdominal cramps, and gas (SURI *et al*., 2019). In this context, the development of a lactose-free probiotic powder would meet this specific population demand.

Probiotic powders obtained by spray drying are usually microparticles in the amorphous state (ARSLAN-TONTUL, 2020; VERRUCK *et al*., 2019; WANG; LIN; ZHONG, 2020). This amorphous structure is associated with the presence of carbohydrates in the dehydration media, and has also been related to the improved stability of spray-dried microorganisms (CHÁVEZ; LEDEBOER, 2007; PASSOT *et al*., 2012; VIVEK; MISHRA; PRADHAN, 2020). Depending on the temperature and relative humidity at which a powder product is subjected, it becomes susceptible to interactions with water, resulting in unwanted characteristics such as agglomeration, difficulty in rehydration, and triggering Maillard reactions. These physical changes can negatively interfere with the microencapsulated bacteria, leading to partial release or mortality. In other words, the physical state of microparticles enhances or minimizes the

probability of chemical changes. Zhu *et al*. (2013) employed whey protein isolate, maltodextrin, D-glucose, and L-glucose as encapsulant matrices of *Lactobacillus rhamnosus* GG. D-glucose, which can be used by probiotic for energy, clearly exerted a physico-chemical action in stabilizing the bacteria. Likewise, the authors noted that water activity (a_w) of the probiotic powders had a large effect on the cell viability during storage. Independently of the rubbery or glassy state of the powders, an increase in a_w carried a quicker reduction in the culturability of *L. rhamnosus* during storage. At a high a_w level (0.70), it was not observed effect of glucose in cell survival, but at 0.33 (a_w most commonly found in dried products), glucose incorporation greatly improved the cell preservation for 35 days compared to a glucose-free carrier. Besides, Romano *et al*. (2018) evaluated the stability of a bacteria with probiotic properties after it was spray-dried with amorphous inulin. The bacteria stability was considered to depend mainly on the a_w in which the powder was stored. Thus, $a_w < 0.40$ resulted in stable cell viability for up to 180 days of storage; whereas, when samples were stored at a_w above this value, microorganisms experienced a significant reduction in their viability. Given all the above, the study of the water adsorption kinetic becomes a valuable tool in the investigation of powders, since this analysis can help in the selection of suitable packaging materials and storage conditions (RHIM; KOH; KIM, 2011). In conjunction with this examination, we also believe that the study of sorption isotherms is essential in identifying optimal storage conditions, as it predicts and models the moisture changes that occur during this period. Consequently, the shelf life of many products can be predicted. Santos and Machado (2021) produced probiotic particles based on alginate–chitosan and studied their sorption isotherms. In addition, the authors used FT-IR analyses to qualitatively examine the presence of bindings in structures generated from the mixtures. Other researchers have also studied the water sorption behavior of probiotic powders produced by spray drying from different carrier agents (AGUDELO *et al*., 2017; GUERGOLETTO; BUSANELLO; GARCIA, 2017; ROMANO *et al*., 2018; VIVEK; MISHRA; PRADHAN, 2020; YING *et al*., 2016).

According to Verruck *et al*. (2018), thermal analysis techniques, such as differential scanning calorimetry and thermogravimetry, can be effectively used to determine the phase transition temperatures in spray-dried powders, as well as the degradation temperatures of its components. Moreover, Pinto *et al*. (2015), Muhammad *et al.* (2017), Dias *et al*. (2018), and De Liz *et al.* (2020) observed a close relationship between the probiotic powders thermal properties and the viable cells count.

In this work, lactose-free milk, oligofructose, and inulin were carefully selected and evaluated in order to know their properties for future food applications. Thus, this study is

intended to assess the physical stability of probiotic powders (based on thermal and water sorption properties analyses), as well as to characterize them in terms of morphology and structure.

2 MATERIAL AND METHODS

2.1 MATERIAL

Lactose-free skim milk powder (Aurora®, Cooperativa Central Aurora Alimentos, Santa Catarina, Brazil) (85.51 g total solids 100 g^{-1}, 32.50 g protein 100 g^{-1}, 0.00 g fat 100 g^{-1}, 3.01 g ash 100 g^{-1}, and 50.00 g carbohydrates 100 g^{-1}) and the prebiotics inulin (Orafti® Gr, Orafti, Tienen, Belgium) (DP ≥ 10) and oligofructose (Orafti® P95, Orafti, Tienen, Belgium) (DP = 2−8) were used as microsphere carrier agents. *Bifidobacterium* BB-12 (Nu-trish® BB-12®, Chr. Hansen, Hønsholm, Denmark) was used as the active material for the microspheres, while UHT (ultra-high temperature) lactose-free milk (3.2 g 100 g^{-1} of proteins, 5.0 g 100 g^{-1} of carbohydrates and 0.40 g 100 g^{-1} of lipids) was employed to prepare the bacterial suspension. The water sorption kinetics and moisture adsorption isotherms were conducted using salts of analytical grade.

2.2 MICROENCAPSULATION OF *BIFIDOBACTERIUM* BB-12 AND VIABLE PROBIOTIC CELL COUNT

Three feed solutions were prepared for the production of spray-dried powders containing *Bifidobacterium* BB-12. Therefore, the feed solutions designated as 1, 2, and 3, presented lactose-free skim milk powder (200 g L^{-1}); lactose-free skim milk powder (100 g L^{-1}) and inulin (100 g L^{-1}); and lactose-free skim milk powder (100 g L^{-1}) and oligofructose (100 g L^{-1}), respectively. All of them were prepared with sterile distilled water and heat-treated for 30 min at 80 °C. After the solutions were cooled down to room temperature (25 °C), a precipitate of probiotic cells was added to each of them. A laboratory-scale spray dryer (B-290 mini spray dryer, Buchi, Flawil, Switzerland) equipped with a cyclone was used. The feed solutions containing *Bifidobacterium* BB-12 were kept under magnetic agitation (MS-3000, BioSan, Riga, Latvia) at room temperature, and spray dried under optimum conditions of 150 °C inlet temperature and 44 °C outlet temperature to obtain probiotic spray-dried powders. The mini spray dryer has an integrated standard two-fluid nozzle, where compressed air is used to

disperse the liquid body into fine droplets. The nozzle consists of a 0.7 mm liquid orifice diameter, a 1.1 mm liquid outer diameter, and a 1.5 mm gas orifice diameter. This geometry results in a mixing of fluid body and gas. Therefore, the feed solution was sprayed by the nozzle in a closed cylindrical container, in which the droplets dry during their fall onto the container wall due to the hot-air flow. The powder and wet air were separated in the cyclone, and then, the sample was collected from the cyclone base. The compressor air pressure, drying airflow rate, and feed rate were set at 0.7 MPa, 35 $m^3\ h^{-1}$, and 12 mL min^{-1}, respectively The powders were denoted as 1, 2, and 3, and were derived from feed solutions 1, 2, and 3, respectively. They were packed in aluminum pouches under vacuum (200 B, Selovac, São Paulo, Brazil).

For enumeration of entrapped cells, 1 g of spray-dried powder was previously vortexed with 9 mL of sterile phosphate buffer (pH 7.0, 0.1 mol L^{-1}) for 10 min. Then, the pour plate method described by Vinderola and Reinheimer (1999) was used. For this, mixtures and feed solutions were serially diluted in peptone water (Oxoid; 0.1 g 100 mL^{-1}), and plated on MRS agar modified with the addition of 0.3 g 100 g^{-1} sodium propionate (Fluka, Neu-Ulm, Germany) and 0.2 g 100 g^{-1} lithium chloride (Vetec, Rio de Janeiro, Brazil). The plates were incubated in anaerobic jars containing AnaeroGen® at 37 °C during 72 h. Results were expressed as log colony-forming units per gram (log CFU g^{-1}).

2.3 WATER SORPTION PROPERTIES

The kinetics of water absorption was studied by fitting the Peleg model to the experimental data. For this, the methodology proposed by Verruck *et al*. (2018) was used. Probiotic spray-dried powders were dehydrated at 105 °C until reaching constant weight, thus, their initial moisture contents (X_0) were measured. After drying, triplicate samples (~ 0.5 g) were placed at 25 ºC in separate desiccators that contained different saturated salt solutions. The saline solutions provided, inside the desiccators, an environment with a relative humidity that varied from 11 to 90%. The sample mass was measured periodically until reaching the hygroscopic equilibrium that was concluded when the sample showed two similar consecutive weightings. The time required to reach that thermodynamic equilibrium varied according to the different relative humidity in which the samples were exposed, that is, 11.25 h in the 11% relative humidity, reaching 6 days for the relative humidity of 80 and 90%. The moisture adsorption curves of the samples were fitted to Equation (1) (PELEG, 1988).

$$X_{(t)} = X_0 + \frac{t}{k_1 + k_2 t} \quad (1)$$

where $X_{(t)}$ is the water content of the powder for a given instant of time (g water / g dry solid), X_0 is the initial moisture of the powder (g water / g dry solid), t is the time (h), k1 is the Peleg rate constant (g dry solid h / g water), and k2 is the Peleg capacity constant (g dry solid / g water).

The moisture adsorption isotherms of the probiotic spray-dried powders previously dehydrated were determined through the static method, using saturated saline solutions to obtain different air relative humidity as described by Labuza, Kaanane, and Chen (1985). The empirical mathematical model of GAB (Equation 2) was employed to retract the experimental equilibrium data (AL-MUHTASEB; McMINN; MAGEE, 2002). The parameters of the model were estimated by nonlinear regression using the STATISTICA 13.3 software (TIBCO Software Inc., Palo Alto, CA).

$$X_{eq} = \frac{M_0\, C\, k\, a_w}{(1 - k\, a_w)(1 - k\, a_w + C\, k\, a_w)} \quad (2)$$

where X_{eq} is the equilibrium moisture (g water / g dry solid), M_0 is the moisture content in the monolayer (g water / g dry solid), a_w is the water activity, and C and k are model constants.

2.4 PHYSICAL CHARACTERIZATION OF PROBIOTIC POWDERS

2.4.1 Morphology and particle size

The morphology and particle size of the spray-dried powders were observed with a Jeol scanning electron microscope, model JSM 6390 LV (Jeol, Tokyo, Japan), at an accelerating voltage of 5 kV. Therefore, the microparticles were fixed with carbon tape on a stub (this was carried out on the day of obtaining the powders) and covered with gold.

The diameter of the powders was estimated from the SEM micrographs in their initial magnification using ImageJ (version 1.51k; http://rsb.info.nih.gov/ij/). The diameter of 120 particles from each of the powders was measured (FRITZEN-FREIRE *et al*., 2012).

2.4.2 X-Ray diffraction

X-ray powder diffraction (XRPD) analysis of the spray-dried powders was performed on an XPERT PANalytical diffractometer, equipped with an X'Celerator detector and using filter radiation of Cu Kα (λ = 1.5418 Å), the tension of 45 kV and current of 40 mA. Samples were analyzed at a scattering range of 4° < 2θ < 60° with 0.12º stepsize and 30 s counting time with at least 5 scans averaged to improve data statistics.

2.4.3 Raman spectroscopic analysis

Raman spectra of spray-dried powders and prebiotics samples were obtained with a PeakSeeker PRO-785 Raman spectrometer using a 50 X objective lens at room temperature. Raman system with a diode laser of 785 nm and 300 mW at the source were employed. Raman spectra were collected at 6 cm^{-1} resolution in the range of 200–2000 cm^{-1} with a Peltier-cooled charge-coupled device CCD detector.

2.4.4 Thermal properties

For obtaining of the thermogravimetry/derivative thermogravimetry (TGA/DrTGA) curves, it was used a DTG-60 thermobalance (Shimadzu DTG-60, Kyoto, Japan) previously calibrated with calcium oxalate. Six milligrams of each spray-dried powder were placed in an aluminum pan. Then, a heating rate of 10 °C min^{-1} was employed to heat the sample from 30 to 300 °C, maintaining a dynamic synthetic air atmosphere of 50 mL min^{-1}.

The spray-dried powders and the raw materials were submitted to differential scanning calorimetry (DSC) (Shimadzu DSC-60, Kyoto, Japan). A standard reference of indium was used for preliminary calibration of equipment. So, approximately two milligrams of sample were placed in aluminum pans covered with a lid. All measurements were performed at 10 °C min^{-1} under a dynamic synthetic air atmosphere of 50 mL min^{-1}, and in a temperature range of 30 to 300 °C.

2.5 STATISTICAL ANALYSIS

The mean and standard deviation (SD) were calculated from data obtained in triplicate. The one-way analysis of variance (ANOVA) was conducted using the STATISTICA version 13.3 software (TIBCO Software Inc., Palo Alto, CA). Differences between treatments mean values were analyzed using the Tukey test at a significance level of 0.05.

3 RESULTS AND DISCUSSION

3.1. PROBIOTIC VIABILITY

The viable probiotic cell count in the feed solutions, and in the spray-dried powders were higher than 10.00 log CFU g^{-1}, and 9.00 log CFU g^{-1}, respectively. Therefore, all spray-dried powders are considered a probiotic product.

3.2 WATER SORPTION PROPERTIES

Peleg's constants at different relative humidity are shown in Table 6. Khazaei and Mohammadi (2009) affirm that the constant k1 is related to the initial rate of absorption, and the lower its value, the higher the initial mass transfer rate. Therefore, we can also study it through its reverse (1/k1). For all samples, the values found for this constant showed sensitive but significant changes ($P < 0.05$) when the relative humidity where the sample was conditioning increased. There was a tendency to increase the values of 1/k1 with the increase of the relative humidity, mainly from 11% for the other relative humidity. This behavior was already expected, as according to Verruck *et al*. (2018), the initial rate of absorption depends of the difference between the moisture content of the sample and the moisture content of the environment (saturation humidity). In other words, the higher the moisture gradient, the higher the initial rate of absorption, since the gradient is the driving force of the process. Besides, these values may be explained due to the high hydrophilicity of the components (carbohydrates and protein) (ZHANG *et al*. 2018). Wang *et al*. (2020) affirmed that at water activity (a_w) between 0 and 0.34, the numerous polar groups of proteins are known as the primary component absorbing water because they can strongly and rapidly absorb water via hydrogen bonding. The high hydration of the casein micelles in their native structure (3.7 mL H_2O g^{-1} protein) contributes to this (SCHCK, 2011).

According to Khazaei and Mohammadi (2009), the constant k2 of the Peleg's model is inversely related to the maximum water adsorption capacity, i.e., the lower the k2, the higher

the water absorption capacity of the product. In our work, 1/k2 values increased with the increase of the relative humidity ($P < 0.05$) (Table 6), especially at the highest relative humidity (80 and 90%). The adsorption of moisture is due to the movement of water (water vapor available to make exchanges with the sample), given the difference in water vapor pressure between the product surface and the air surrounding it. Consequently, from the moment that we increase the relative humidity inside the desiccator, the vapor pressure of air also increases, which explains the values of 1/k2 found. Spray-dried powder 1 sample showed the highest value of 1/k2 at 90% relative humidity (0.6936), followed by spray-dried powder 3 (0.5706), and finally, spray-dried powder 2 (0.4860). As occurred in the work by Verruck *et al*. (2018), higher values of 1/k2 were correlated with the highest equilibrium moistures observed during the experiments, since the equilibrium moistures of the samples when subjected to the environment with 90% relative humidity were 0.6551, 0.5295, and 0.4637 g water g^{-1} dry solid for spray-dried powders 1, 3 and 2, respectively. Our results for all samples are also in agreement with those obtained by Vivek, Mishra, and Pradhan (2020), who studied probiotic spray-dried powder obtained from Sohiong fruit. In both cases, the equilibrium moisture content increased as the relative humidity increased.

The higher values of 1/k2 constant and equilibrium moisture for spray-dried powder 1 may be explained by the higher amount of short-chain carbohydrates glucose and galactose, they are simpler carbohydrates compared to the prebiotics used in the other preparations. Saavedra-Leos *et al*. (2014) reported that more water molecules can be readily absorbed by these carbohydrates, given a higher content of OH groups on their surfaces. Moreover, Jimenez-Sánchez *et al*. (2018) highlighted that the lower the degree of polymerization of a product, the more hygroscopic it will be. On the other hand, Pilatti-Riccio *et al*. (2019) reported that, given the high hygroscopicity, the use of short-chain carbohydrates in mixtures of wall materials is advantageous because it can result in a fast release of encapsulated compounds. Another factor that is closely related to the moisture and water activity of a sample is the glass transition temperature (T_g). Galactose and glucose present lower T_g than oligofructose and inulin (30, 31, 102, and 132 ºC, respectively) (SCHUCK *et al*., 2005; SILVA *et al*., 2016; HINRICHS; PRINSEN; FRIJLINK, 2001). Juliano and Barbosa-Cánovas (2010) discussed that a glass transition temperature nearby to room temperature favors water absorption, and as consequence, it can bring technological problems to milk powder such as rehydration difficulty, particle agglomeration, and cacking.

Table 6 – Peleg model coefficients and fit parameters. 1/k1 = g water. (g dry solid. h) $^{-1}$; 1/k2 = g water. (g dry solid) $^{-1}$.

Samples	% RH	1/k1	1/k2	R^2	SSE
Spray-dried powder 1	11	0.0186 ± 0.0057^{bA}	0.0266 ± 0.0007^{fA}	0.9676 ± 0.0061^{cB}	4.02×10^{-5}
	33	0.0451 ± 0.0074^{aA}	0.0741 ± 0.0018^{eAB}	0.9840 ± 0.0041^{bB}	1.46×10^{-4}
	43	0.0408 ± 0.0042^{abA}	0.0952 ± 0.0010^{eA}	0.9917 ± 0.0020^{abA}	1.69×10^{-4}
	58	0.0556 ± 0.0039^{aA}	0.1487 ± 0.0018^{dA}	0.9934 ± 0.0003^{abA}	3.49×10^{-4}
	75	0.0478 ± 0.0031^{aA}	0.2787 ± 0.0074^{cA}	0.9975 ± 0.0005^{aA}	4.29×10^{-4}
	80	0.0583 ± 0.0089^{aA}	0.3802 ± 0.0206^{bA}	0.9965 ± 0.0008^{aA}	1.78×10^{-3}
	90	0.0427 ± 0.0064^{aA}	0.6936 ± 0.0106^{aA}	0.9920 ± 0.0014^{abA}	1.46×10^{-2}
Spray-dried powder 2	11	0.0144 ± 0.0005^{bA}	0.0262 ± 0.0019^{gA}	0.9892 ± 0.0019^{aA}	1.18×10^{-5}
	33	0.0356 ± 0.0091^{abA}	0.0674 ± 0.0025^{fB}	0.9927 ± 0.0025^{aAB}	5.22×10^{-5}
	43	0.0359 ± 0.0061^{abA}	0.0921 ± 0.0003^{eA}	0.9895 ± 0.0033^{aA}	2.03×10^{-4}
	58	0.0348 ± 0.0022^{abB}	0.1431 ± 0.0002^{dA}	0.9954 ± 0.0003^{aA}	2.27×10^{-4}
	75	0.0524 ± 0.0071^{aA}	0.2390 ± 0.0019^{cB}	0.9960 ± 0.0029^{aA}	5.38×10^{-4}
	80	0.0500 ± 0.0057^{aA}	0.2850 ± 0.0012^{bB}	0.9964 ± 0.0002^{aA}	1.06×10^{-3}
	90	0.0403 ± 0.0023^{aA}	0.4860 ± 0.0074^{aC}	0.9933 ± 0.0020^{aA}	5.93×10^{-3}
Spray-dried powder 3	11	0.0100 ± 0.0001^{cA}	0.0241 ± 0.0004^{fA}	0.9877 ± 0.0034^{aA}	1.09×10^{-5}
	33	0.0182 ± 0.0024^{bcA}	0.0766 ± 0.0003^{eA}	0.9981 ± 0.0008^{aA}	1.46×10^{-5}
	43	0.0279 ± 0.0005^{abA}	0.0963 ± 0.0024^{eA}	0.9910 ± 0.0012^{aA}	1.92×10^{-4}
	58	0.0315 ± 0.0045^{abB}	0.1543 ± 0.0094^{dA}	0.9908 ± 0.0075^{aA}	5.42×10^{-4}
	75	0.0387 ± 0.0030^{aA}	0.2767 ± 0.0022^{cA}	0.9983 ± 0.0004^{aA}	2.75×10^{-4}
	80	0.0336 ± 0.0054^{aA}	0.3497 ± 0.0032^{bA}	0.9983 ± 0.0010^{aA}	7.56×10^{-4}
	90	0.0306 ± 0.0056^{abA}	0.5706 ± 0.0088^{aB}	0.9954 ± 0.0004^{aA}	5.57×10^{-3}

$^{a–g}$ Means ± standard deviation with different superscript lowercase letters in the same column indicate significant differences ($P < 0.05$) among the different relative humidity (RH) for each sample. $^{A–C}$Means ± standard deviation with different superscript uppercase letters in the same column indicate significant differences ($P < 0.05$) among the samples on the same relative humidity (RH). Means found in triplicate.

Chirife *et al*. (1992) stated that a mechanistic approach is needed for a full-proof validation, that is, a mere fitting of the sorption model to the experimental data cannot guarantee its validity. We concluded that the Peleg model fits exceptionally well to the experimental data because, in addition to showing high values of R^2 and low values of SSE (Table 6), randomness in the standardized residual plots was observed in all cases. Moreover, it was noticed a good fit in the highest, intermediate, and the lower relative humidity, providing adequate values of the initial rates of water adsorption and maximum water adsorption capacity. Given its relative simplicity, Peleg's model has been used satisfactorily by several authors to represent hydration kinetics in dairy products (SETH *et al.*, 2018; RUANO-USCATEGUI; CIRO-VELÁSQUEZ; SEPÚLVEDA-VALENCIA, 2018; VERRUCK *et al*., 2018; LIANG; BUND; HARTEL, 2009; VARGHESE, RAMACHANDRANNAIR; MISHRA, 2009).

Vivek *et al*. (2020) emphasized that data obtained from the isotherm study can be useful to define process condition, transport, and storage, to predict desorption or adsorption behavior of the powder and the shelf life of the material. It can also be used to describe the energy requirements of a dehydration process. Experimental data of adsorbed moisture content (equilibrium moisture) as a function of a_w was well described by the Guggenheim–Anderson–Boer (GAB) model (Fig. 3). The data for all probiotic powders followed a type III isotherm behavior. In addition to indicating the formation of multilayer, the type III isotherm curve is related to the presence of amorphous sugars and their dissolution in water (RAO; RIZVI, 1994).

Reid and Fennema (1996) stated that the isotherms are classified into three regions, as follows: first, monolayer moisture region, which includes a_w less than 0.2 to 0.3; second, multilayer moisture region, which comprises a_w between about 0.2 and 0.3 until approximately 0.8; and third, free water region, that represents the most mobile and least bound water in foods. In region III, a_w are equals to 0.8–0.99, and it was in this region that the samples showed a drastic increase in the adsorption of water. According to Verruck *et al*. (2018), in this region, the product can undergo some microbiological alteration, chemical, or biochemistry because of the available water.

The coefficients of the GAB model were presented in Table 7. The coefficient of determination (R^2) and Sum of Squares Error (SSE) indicate excellent fit of the model to the experimental data, due to their proximity to 1 and 0, respectively. Moreover, the distribution of the residuals was randomly around zero. M_0 value means the monolayer moisture content on dry basis, and it indicates a strong binding potency of water on the surface of the product if moisture content remains above this value. Therefore, this information is vital for the storage stability of food products. The values of parameter C represent the heat of sorption of monolayer

moisture while the parameter k provides the binding potency of water in terms of heat of sorption of multilayer moisture. For all of our spray-dried powders, the values of parameter C were higher than those values of k. Seth *et al*. (2018) also noted similar behavior for spray dried yogurt powder. The k values were lower ($P < 0.05$) for the spray-dried powders 2 and 3 than in the spray-dried powder 1 sample. Ronkart *et al.* (2006) and Verruck *et al*. (2018) reported the same behavior, they found lower k values for samples that contained prebiotics (higher molar mass).

Figure 3 – Moisture sorption isotherms of spray-dried powders that contain *Bifidobacterium* BB-12. (a) Spray-dried powder 1 produced with the carrier agent skim lactose free milk; (b) Spray-dried powder 2 produced with the carrier agents skim lactose-free milk and inulin; and (c) Spray-dried powder 3 produced with the carrier agents skim lactose free milk and oligofructose. The GAB model is the solid line ($R^2 > 0.99$).

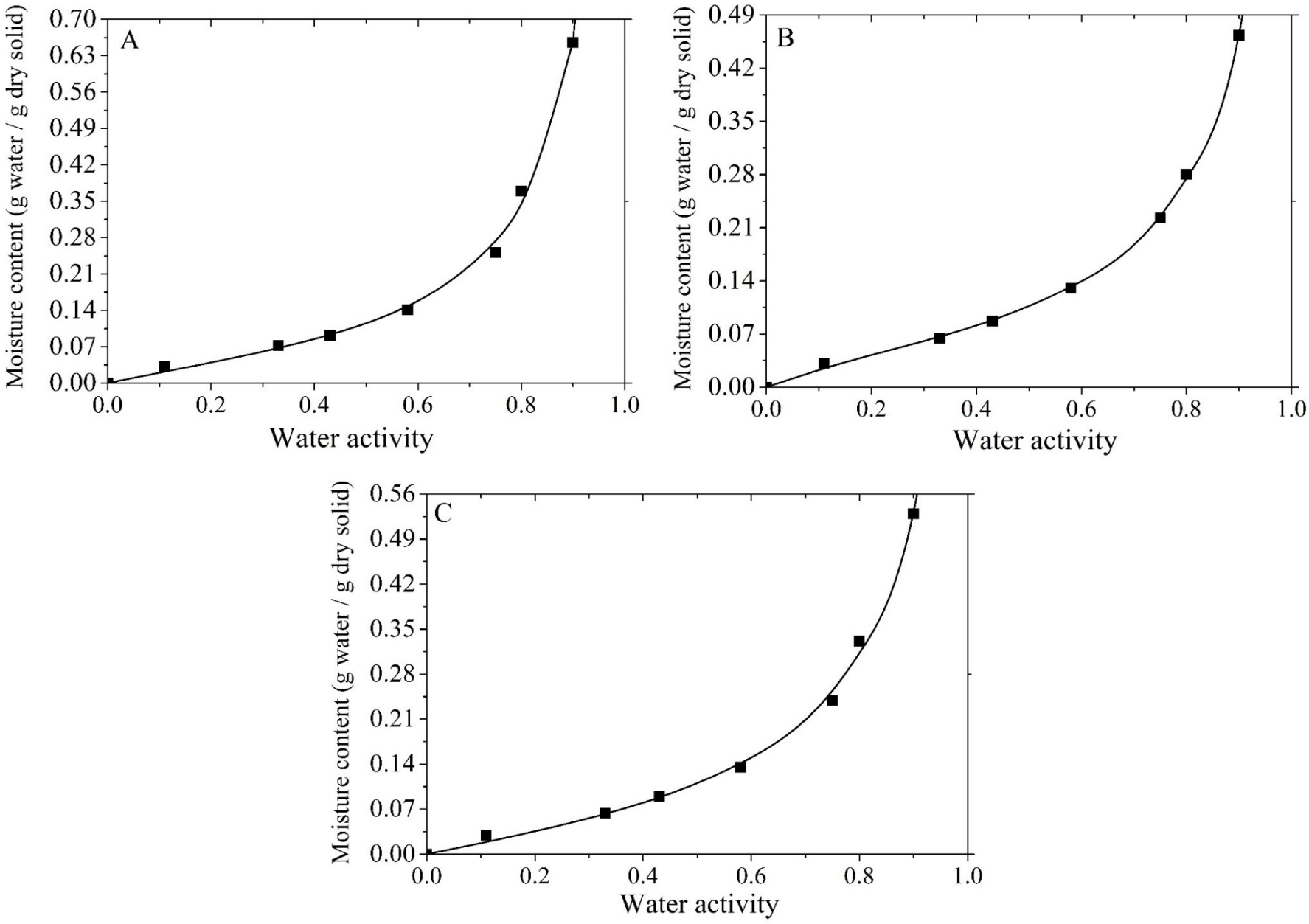


Table 7 – GAB model coefficients and fit parameters. M_0 = g water. (g dry matter) $^{-1}$.

	Spray-dried powder 1	Spray-dried powder 2	Spray-dried powder 3
M_0	0.084 ± 0.005^{ab}	0.073 ± 0.004^{b}	0.092 ± 0.001^{a}
C	2.573 ± 0.583^{a}	3.786 ± 0.775^{a}	1.994 ± 0.018^{a}
k	0.977 ± 0.005^{a}	0.944 ± 0.008^{b}	0.935 ± 0.005^{b}
R^2	0.998 ± 0.002^{a}	0.999 ± 0.001^{a}	0.998 ± 0.001^{a}
SSE	0.00143	0.00016	0.00072

3.3 PHYSICAL CHARACTERIZATION OF PROBIOTIC POWDERS

3.3.1 Morphology and particle size

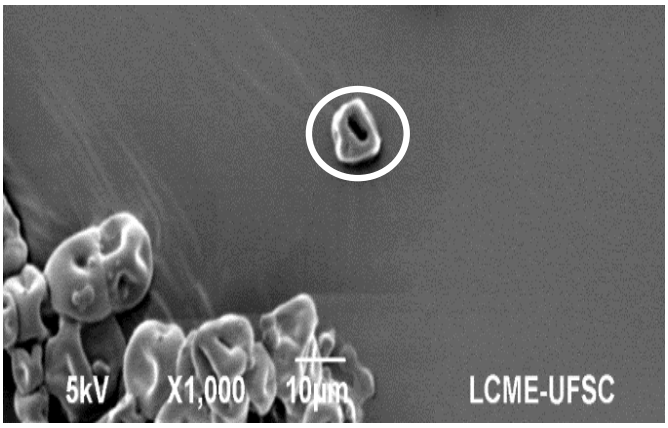
Fig. 4 shows the SEM micrographs of the *Bifidobacterium* BB-12 microspheres produced with different wall materials. Micrographs revealed distinct shapes and sizes for each spray-dried powder; however, in all of them, it is evident that *Bifidobacterium* BB-12 cells were retained therein because it was possible to note the absence of cells on the outside of their surfaces. The particles produced with lactose-free milk (Fig. 4-a) showed concavities typical of materials produced by spray drying. Gul (2017) reported that the formation of concavities in the surface of atomized particles can be attributed to the shrinkage of the particles during the drying process because of the rapid evaporation of the liquid drops. Similar morphological shapes of capsules made with reconstituted skim milk were reported by Maciel *et al*. (2014). The spray-dried powder 2, produced with lactose-free milk powder and inulin, showed a rough and uneven surface, however, free of fissures or disruptions, which is fundamental for guaranteeing higher protection and lower permeability of gases (FRITZEN-FREIRE *et al*., 2012). Microspheres produced with oligofructose and lactose-free milk (Fig. 4-c) were spherical, with more aggregation than spray-dried powders 1 and 2. This aggregation of particles is due to stickiness caused by the low glass transition temperature of oligofructose (ADHIKARI *et al*., 2009). The same behavior was observed with the microcapsules produced by Rajam and Anandharamakrishnan (2015), who microencapsulated *Lactobacillus plantarum* with fructooligosaccharide as wall material.

Knowledge of the bulk density is important during the processing, storage, and packaging of encapsulated microparticles. The parameters such as moisture content, particle size distribution, and morphology can affect the bulk density of spray-dried powders (RAJAM;

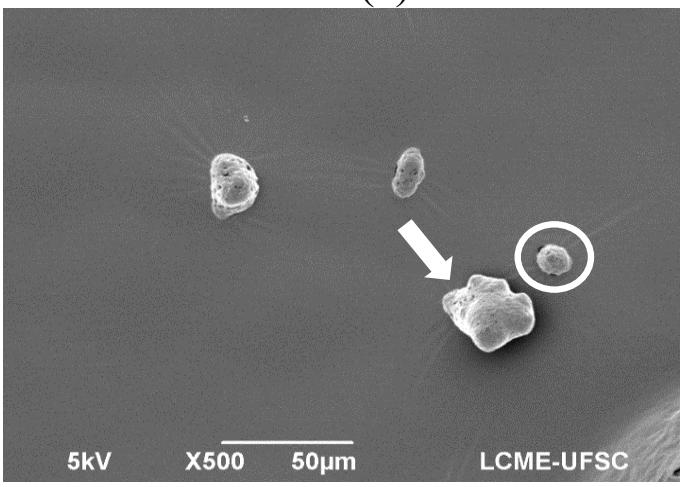
ANANDHARAMAKRISHNAN, 2015). In our previous work (DANTAS *et al*., 2021), we measured the loose bulk density of the powders. It was possible to verify that the spray-dried powder 3 showed a significant variation ($P < 0.05$) of this property (0.44 g cm^{-3} against 0.32 g cm^{-3} for both spray-dried powders 1 and 2). These results reinforce our considerations about micrographs, and we associate them with particle aggregation and less interspace between particles of the spray-dried powder 3.

The size is an important property for the microparticle because of its strong influence on product solubility, appearance, and acceptability (PARTHASARATHI; ANANDHARAMAKRISHNAN, 2016). In the present study, the probiotic spray-dried powders microparticles exhibited a wide range of average diameters: 10.62 ± 4.94, 19.60 ± 7.52, 55.66 ± 21.69 μm, for spray-dried powder 1, 2, and 3 respectively. This microparticle size is favorable for the possible incorporation of the powders in various matrices without significantly affecting the texture. According to Turchiuli *et al*. (2011), stable solid bridges between particles can be created with the drying, leading to the formation of a bigger structure that is clustered, as observed by SEM images (Fig. 4-b,c). The presence of clusters in the spray-dried powder 2 and 3 corroborate with results obtained for the average size of the microparticles. The cluster structures visualized in the present study could be formed by several particles bound together. Carmo *et al*. (2018) discussed that the difference between the particle sizes is characterized by droplet coalescence, which in turn is influenced by the carrier agent used. According to Berdnaska and Janiszewska-Turak (2020), the use of carriers' agents or their mixtures in different proportions can result in powders with different physical properties. In the present study, no changes were made in the spray drying processes; therefore, encapsulation efficiency may have been influenced by the carrier agents. Ronkart *et al*. (2009) highlighted that in the spray drying process, two associated factors can generate the amorphous state of inulin and oligofructose and, therefore, both would be more susceptible to the clusters formation. These factors are the temperature (such as inlet temperature) and the water present in the droplets formed.

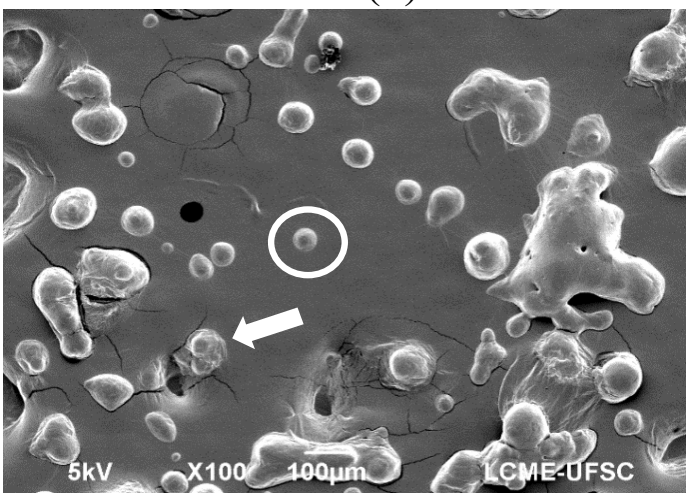
Figure 4 − Micrograph of spray-dried powders that contain *Bifidobacterium* BB-12. (a) Spray-dried powder 1 produced with the carrier agent skim lactose free milk; (b) Spray-dried powder 2 produced with the carrier agents skim lactose-free milk and inulin; and (c) Spray-dried powder 3 produced with the carrier agents skim lactose free milk and oligofructose. Circle and arrow in white color represent the microsphere and the cluster, respectively.



(a)



(b)



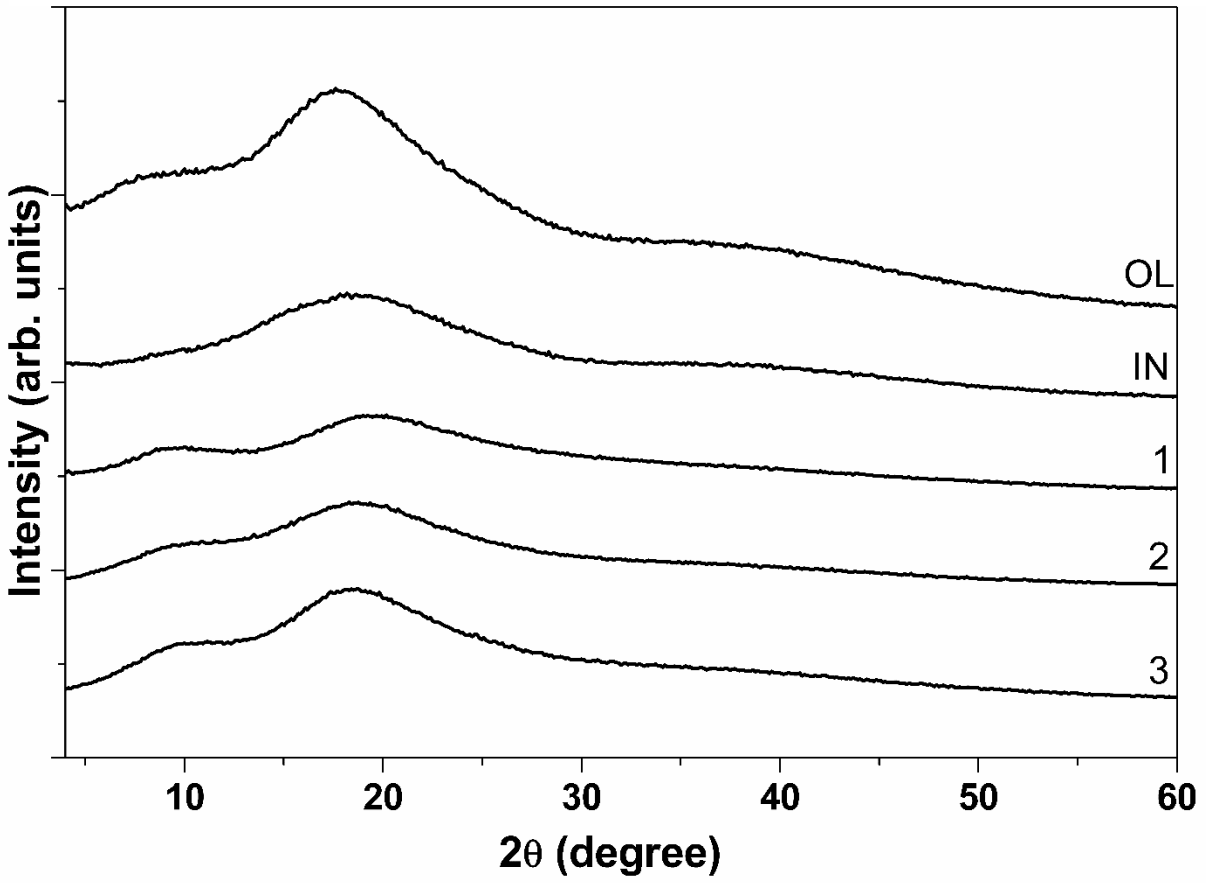
(c)

3.3.2 X-Ray diffraction

The diffractograms of the samples (spray-dried powders, inulin, and oligofructose) are shown in Figure 5. This analysis can be utilized to characterize the type of order present in powders. X-ray amorphous materials lack long-range crystallographic order and produce a broad background pattern. The crystalline solids present long-range order and their diffractograms show a series of sharp peaks (AZÁROFF, 1968). The diffraction pattern obtained for the spray-dried powders which contain *Bifidobacterium BB-12* microspheres is predominantly related to amorphous material, since it shows dispersed bands, indicating that the molecules are disordered. De Medeiros *et al*. (2014) also obtained similar results, they studied spray drying dehydration of a probiotic yogurt produced with goat's milk and *Bifidobacterium animalis* subsp. *lactis* (BI-07). According to the same authors, the crystals could damage the cells, which would reduce the viability of microorganisms, making the amorphous structure interesting. Besides, amorphous solids are in general more soluble, and the crystallization may entail a negative impact on the handling properties (CAMPELO *et al*., 2017).

Campelo *et al*. (2017) used oligofructose (DP = 2−10) and inulin (DP= 2−60) as wall material to microencapsulate lime essential oil by spray drying. As with our results, they also found an amorphous type structure for these types of prebiotics, revealing that the spray drying process did not influence their structures. This is probably associated with the rapidity of the drying process, which prevents easy crystallization. Other studies have also reported amorphous characteristic for inulin with different polymerization degrees (KALAIVANI; SUJA, 2018; SILVA; MEIRELES, 2015), for oligofructose (ALLES; TESSARO, NORENA, 2013), and spray-dried skim milk powder (YAZDANPANAH; LANGRISH, 2016).

Figure 5 − Diffractograms of wall materials and spray-dried powders that contain *Bifidobacterium* BB-12. (OL): oligofructose, (IN): inulin, (1): Spray-dried powder produced with the carrier agent skim lactose free milk, (2) Spray-dried powder produced with the carrier agents skim lactose-free milk and inulin, and (3) Spray-dried powder produced with the carrier agents skim lactose free milk and oligofructose.



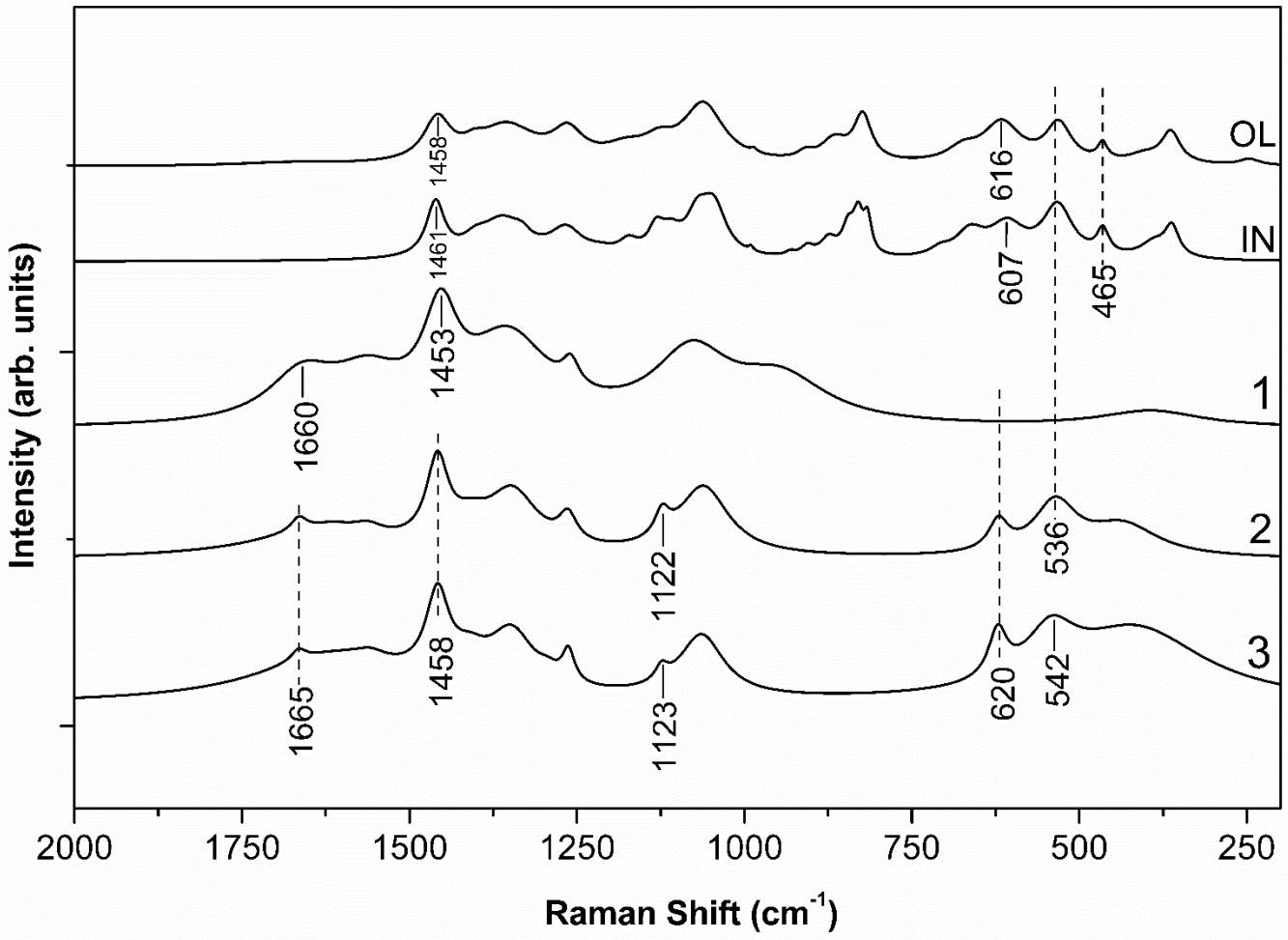
3.3.3 Raman spectroscopic analysis

Raman spectra of spray-dried powders and prebiotics (oligofructose and inulin) are shown in Fig. 6, and the main vibrational bands are listed in Table 8 with their respective tentative assignments based on comparisons with previously reported data. The spray-dried powder 1 showed a spectrum with typical bands associated with proteins and carbohydrates (glucose). The band at 1660 cm^{-1} was the contribution from the C=O stretching and N–H wagging modes of the group Amide I ($C{=}O{-}NH_2$) (ALMEIDA *et al*., 2011; LI-CHAN, 1996). In the spray-dried powders 2 and 3, this band shifted by 5 cm^{-1} and corresponds to the same vibrations.

A strong Raman band at 1453 cm^{-1} was noted in spray-dried powder 1, and it is related to bending vibrations in the form scissoring of the group CH_2, which was mainly due to the carbohydrate mode (ALMEIDA *et al*., 2011), principally glucose (CERCHIARO *et al*., 2005). Torres *et al*. (2017) also comment that the region between 1500 and 1250 cm^{-1} corresponds to CH_2OH deformation modes. The same peak was observed in the spectra of the other spray-

dried powders, as well as inulin and oligofructose, but with a small shift in its wavenumbers. In the prebiotics spectra, especially oligofructose, this band appeared with lower intensity because of the lower contribution of glucose in these samples.

Figure 6 − Raman spectra of wall materials and spray-dried powders that contain *Bifidobacterium* BB-12. (OL): oligofructose, (IN): inulin, (1): Spray-dried powder produced with the carrier agent skim lactose free milk, (2) Spray-dried powder produced with the carrier agents skim lactose-free milk and inulin, and (3) Spray-dried powder produced with the carrier agents skim lactose free milk and oligofructose.



The bands at 1259, 1263, and 1262 cm^{-1} in the spray-dried powders 1, 2, and 3, respectively, may be related to the contributions from the N–H bending and C–N stretching modes of the group Amide III (LI-CHAN, 1996). As there are no proteins in oligofructose and inulin, these same bands (1263 and 1266 cm^{-1}, respectively) are assigned to the CH_2 twisting mode of carbohydrates (ALMEIDA *et al*., 2011; RODRIGUES JUNIOR *et al*., 2016).

The region between 1121 and 1065 cm^{-1} is assigned to C–O and C–C stretching, and C–O–H bending (RODRIGUES JUNIOR *et al*., 2016). Note that inulin and oligofructose favored the appearance of these vibrations, since the peaks at 1122 and 1123 were observed in the spray-dried powders 2 and 3, respectively; in contrast, this same band was not observed in spray-dried powder 1. According to Almeida *et al*. (2011), these vibrational modes are characteristic of carbohydrates.

The spectra of spray-dried powders 2 and 3 showed two more features that were not observed in the spray-dried powder 1. The peak located at 620 cm^{-1} corresponds to the deformations of groups O–C–O and O–H, this result confirms the presence of prebiotics, since typical bands of inulin are in the spectral region between 833 and 599 cm^{-1} (BALAN *et al*., 2018). Similarly, the band at 536 cm^{-1} was observed for spray-dried powder 2 and at 542 cm^{-1} for spray-dried powder 3, indicating glucose ring deformations of the inulin. Spectra of inulin and oligofructose were very similar, which was already expected since these carbohydrates have the same structure, changing only the molar mass. For example, both spectra show a medium peak at 465 cm^{-1}, being attributed to O–H bending mode (BALAN *et al*., 2018). According to Oroian, Ropciuc, and Paduret (2018), the band at 607 cm^{-1} presents in the inulin spectrum is assigned to the presence of fructose.

Table 8 – Main Raman wavenumbers, in cm^{-1}, and their respective tentative assignments.

Sample	Raman shift (cm^{-1})	Assignment	Reference
Spray-dried powder 1	1259.15	δ (N–H) Amide III; ν (C–N) Amide III; and/or γ $(CH_2)_{twisting}$	(Li-Chan, 1996; Rodrigues Junior *et al*., 2016)
	1352.03	Glucose	(Torres *et al*., 2017)
	1453.83	δ $(CH_2)_{scissoring}$	(McGoverin, Clark, Holroyd, & Gordon, 2010)
	1660.4 m	ν (C=O) Amide I; γ $(N–H)_{wagging}$ Amide I	(Almeida *et al*., 2011; Li-Chan, 1996)
Spray-dried powder 2	536.127 vs	glucose ring def	(Balan *et al*., 2018)
	620.835 s	def (O–C–O); def (O–H)	(Balan *et al*., 2018)
	1122.97 m	ν (C–O); ν (C–C); δ (C–O–H)	(Rodrigues Junior *et al*., 2016)
	1263.39 m	δ (N–H) Amide III; ν (C–N) Amide III; and/or γ $(CH_2)_{twisting}$	(Li-Chan, 1996; Rodrigues Junior *et al.*, 2016)
	1665.1	ν (C=O) Amide I; γ $(N–H)_{wagging}$ Amide I	(Almeida *et al*., 2011; Li-Chan, 1996)
Spray-dried powder 3	621.938 s	def (O–C–O); def (O–H)	(Balan *et al*., 2018)
	1064.11 m	ν (C–O); ν (C–C); δ (C–O–H)	(Rodrigues Junior *et al*., 2016)
	1123.69 m	ν (C–O); ν (C–C); δ (C–O–H)	(Rodrigues Junior *et al*., 2016)
	1262.77 m	δ (N–H) Amide III; ν (C–N) Amide III; and/or γ $(CH_2)_{twisting}$	(Li-Chan, 1996; Rodrigues Junior *et al*., 2016)
	1665.8	ν (C=O) Amide I; γ $(N–H)_{wagging}$ Amide I	(Almeida *et al*., 2011; Li-Chan, 1996)

Sample	Raman shift (cm^{-1})	Assignment	Reference
Oligofructose	465.421	δ (O–H)	(Balan *et al*., 2018)
	531.073 vs	glucose ring def	(Balan *et al*., 2018)
	616.226 s	def (O–C–O); def (O–H)	(Balan *et al*., 2018)
	674.193 m	def (O–C–C); def (O–H); def (C–H)	(Balan *et al*., 2018)
	823.398 s	δ (O–H); def (C–H)	(Balan *et al*., 2018)
	1458.65	$\delta\ (CH_2)_{scissoring}$	(McGoverin *et al*., 2010)
Inulin	464.955	δ (O–H)	(Balan *et al*., 2018)
	532.485 vs	glucose ring def	(Balan *et al*., 2018)
	607.886	Fructose	(Oroian *et al*., 2018)
	663.164 m	def (O–C–C); def (O–H); def (C–H)	(Balan *et al*., 2018)
	707.939 w	def (O–H)	(Balan *et al*., 2018)
	816.277 vs	def (C–C–O); def (O–C–C); δ (C–H)	(Balan *et al*., 2018)
	830.212 s	δ (O–H); def (C–H)	(Balan *et al*., 2018)
	1461.17	$\delta\ (CH_2)_{scissoring}$	(McGoverin *et al*., 2010)

Vs – very strong; s – strong; m – medium; w – weak.

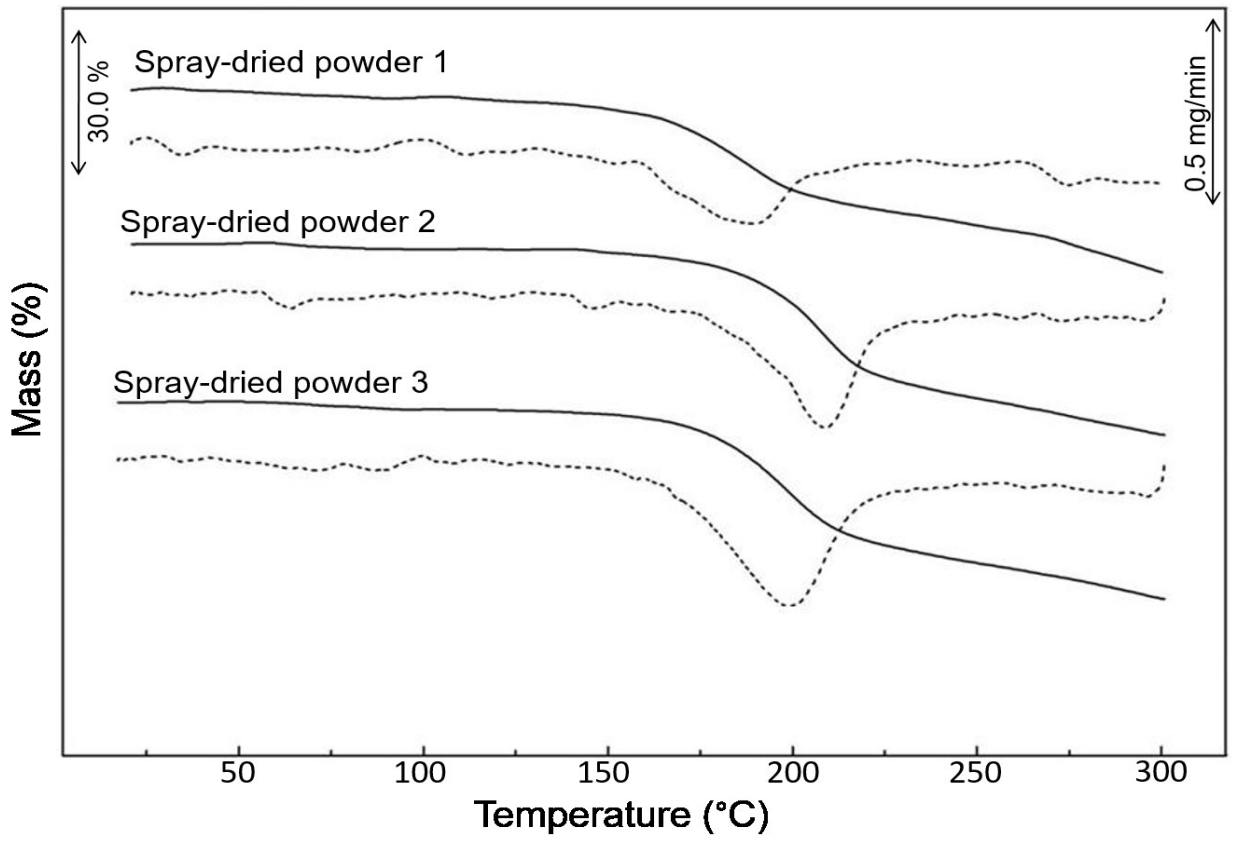
3.3.4 Thermal properties

Figure 7 shows the thermogravimetric (TGA) and derivative thermogravimetry (DrTGA) curves obtained from spray-dried powders. The first weight loss (3.1%, 2.34%, and 2.6% for spray-dried powder 1, 2, and 3, respectively) occurred between 25 °C and 131 °C and it corresponds to the removal of moisture of the samples. Above this temperature range, the decomposition process occurred in one or two stages, according to each sample. Spray-dried powder 1 showed two stages of decomposition: the first between 131 ºC and 233 ºC (mass loss of 24.84%), which is related to the degradation and/or caramelization of the glucose (SAAVEDRA-LEOS *et al*., 2012); and the second stage in the range of 233 ºC to 300 ºC (mass loss of 26.45%) associated with the whey protein degradation and caseins denaturation (BARRETO; PIRES, SOLDI, 2003).

For both spray-dried powders 2 and 3, the decomposition process occurred in only step (temperature range between 131 and 300 °C). However, the DTG_{peak} temperature was higher for spray-dried powder 2 than for spray-dried powder 3 (209.26 and 199.86 °C, respectively), indicating greater stability for inulin-based microcapsules. This behavior was already expected since inulin has a higher degree of polymerization than oligofructose and, therefore, tends to be more thermally stable (VERRUCK *et al*., 2017). Similar results were also observed by Verruck *et al*. (2018) and de Liz *et al*. (2020), who microencapsulated *Bifidobacterium* BB-12 in full-fat goat's milk, whey freeze concentrate, and prebiotics (inulin and oligofructose). These DTG_{peak} temperatures may correspond to the breakdown of the fructose chains of the prebiotics (FRITZEN-FREIRE *et al*., 2012).

When investigating the DSC curve of the wall material Skim Lactose-free Milk (Fig. 8-a), a change in baseline between ~80 and 150 °C indicated us an endothermic event. For this event, the peak mid-temperature was found at 120 ºC. Szulc *et al.* (2016) found peaks around 100 °C for dairy powders, and attributed them to the caseins denaturation. Likewise, De Liz *et al*. (2020) observed endothermic peaks around 110 °C for cryoconcentrated whey samples, which were related to the denaturation of its proteins. O'Mahony *et al*. (2017) reported that at temperatures above 115 °C, protein denaturation peaks could not be distinguished, which was credited to the Maillard reaction induced during the analysis. However, for all spray-dried powders resulting from this sample (Fig. 6-b), there was a well-defined endothermic event, also associated with both non-enzymatic browning (advanced Maillard reaction) (VUATAZ; MEUNIER; ANDRIEUX, 2010) and denaturation of whey proteins (ZHOU; LABUZA, 2011).

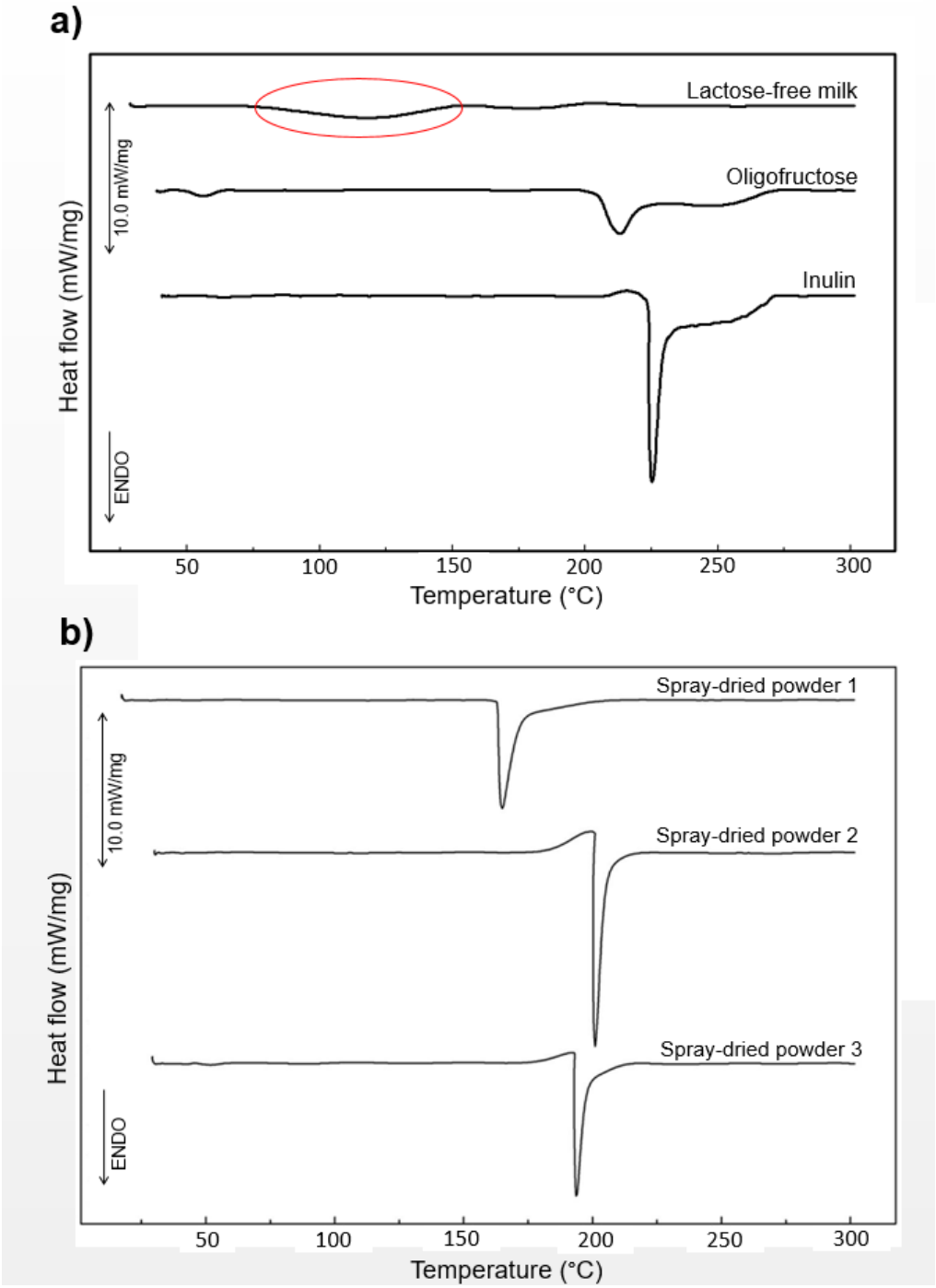
Figure 7 − TGA (__) (solid line) and DrTGA (_ _ _) (dashed line) curves of spray-dried powders that contain *Bifidobacterium* BB-12.



Zhou and Labuza (2011) mentioned that at 53 °C the proteins are barely impacted by the temperature; however, the DNA of the microorganism present in the milk powder matrix can be potently affected leading to damage and cell inactivation. The same authors also commented that the denaturation temperature of whey protein isolate and the β-lactoglobulin (one of the major components of whey proteins) has a strong dependence on the water content of the sample: it decreases with increasing water content. This relation is best visualized when comparing the moisture (dry basis) of the powders on the day of their manufacture with the endothermic peaks: moisture values (g 100 g^{-1}) of 7.67, 4.45, and 4.54, and peak temperatures at 165.02 °C (ΔH = −166.94 J g^{-1}), 200.93 °C (ΔH = − 160.37 J g^{-1}), and 193.70 °C (ΔH = − 111.06 J g^{-1}) for spray-dried powders 1, 2 and 3, respectively. Furthermore, this increase of the peaks in the spray-dried powders 2 and 3 is related to the increase of the thermal stability due to the addition of the prebiotics (PINTO *et al*., 2015). Pilatti-Riccio *et al*. (2019) noted that particles prepared with oligofructose had fewer thermal events in their DSC curves in comparison to the events observed when only the core material was investigated. According to them, this thermal behavior suggests the protection and interaction of the wall material with the core extract, demonstrating that this matrix has potential for application as wall material in the food industry.

As can be seen in Fig. 8-a, the T_{peaks} found for inulin and oligofructose are 225.15 and 211.27 °C, and enthalpy changes −83.92 and −46.84 J g^{-1}, respectively. These peaks can be attributed to the inulin thermal degradation (DAN; GHOSH; MOULIK, 2009; RONKART *et al*., 2010; LEONE *et al*., 2014), as well as caramelization and decomposition of the oligofructose (BERSANET *et al*., 2016). Pilatti-Riccio *et al*. (2019) also observed an endothermic peak at 201.6 ºC for commercial oligofructose.

Figure 8 −DSC curves of raw materials (a) and spray-dried powders that contain *Bifidobacterium* BB-12 (b). The red circle includes the region of a less intense peak observed for lactose-free milk.



Based on these results, it can be stated that the microencapsulation process using inulin or oligofructose conferred greater stability to spray-dried powders. Moreover, it has been found that, during the spray drying process at the air inlet temperature of 150 °C, the materials employed in the production of the microspheres did not suffer any significant degradation.

4 CONCLUSIONS

Bifidobacteria was efficiently entrapped in lactose free milk, and lactose free milk and prebiotics. Peleg model provided fair values of the initial mass transfer rate and water adsorption capacity. The isotherm found for all samples was a Type III Isotherm, it is related to the presence of amorphous sugars, as observed in the powders diffractograms. The GAB model fitted well to the experimental data, and indicated that relative humidity conditions above 33% and 43% were not efficient to maintain the storage stability of the spray-dried powders 1 and 2, and spray-dried powder 3, respectively. The Raman spectrum confirmed the incorporation of the prebiotics in the resultant spray-dried powders (that is, after microencapsulation process), ensuring their availability to the consumer. Besides, the addition of these carbohydrates conferred greater thermal stability to spray-dried powders, especially the inulin. On this approach, we concluded that a good quality of lactose-free milk based probiotic powder could be obtained using spray drying technique, with potential application in the food industry.

Declaration of competing interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Coordination of Improvement of Higher Education Personnel (CAPES, Brazil) [CAPES-PRINT, Project number 88887.310560/2018-00]; to National Council for Scientific and Technological Development (CNPq, Brazil) by the financial support [CNPq, 405965/2016-8], and to Coordination of Improvement of Higher Education Personnel

(CAPES, Brazil) by the scholarship [001]. The authors are also grateful to FAPESC by the financial support. The XRPD and SEM/EDS measurements were performed at Laboratório de Difração de Raios-X (LDRX) and Laboratório Central de Microscopia Eletrônica (LCME) multiuser facilities at UFSC.

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CAPÍTULO 4

Encapsulated *Bifidobacterium* BB-12 addition in a concentrated lactose-free yogurt: its survival during storage and effects on the product's properties

*** Artigo submetido na revista Food Research International**

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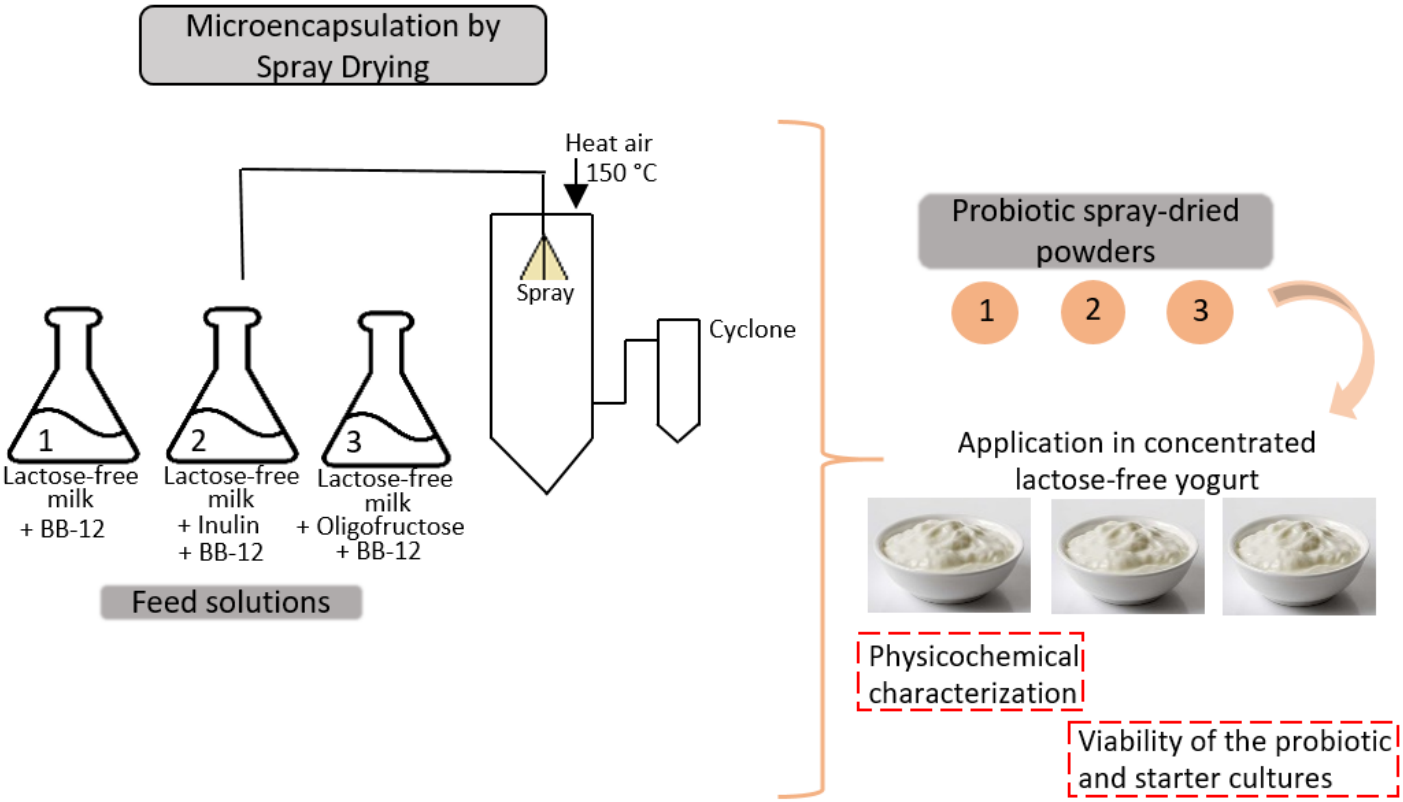

Microencapsulation by Spray Drying
Heat air
150 °C
Spray
Cyclone
1
Lactose-free milk + BB-12
2
Lactose-free milk + Inulin + BB-12
3
Lactose-free milk + Oligofructose + BB-12
Feed solutions
Probiotic spray-dried powders
1
2
3
Application in concentrated lactose-free yogurt
Physicochemical characterization
Viability of the probiotic and starter cultures

Encapsulated *Bifidobacterium* BB-12 addition in a concentrated lactose-free yogurt: its survival during storage and effects on the product's properties

ABSTRACT

This work aims to manufacture a new concentrated lactose-free probiotic yogurt. For this purpose, the probiotic *Bifidocaterium* BB-12 was incorporated in a concentrated lactose-free yogurt, both in its free form and previously encapsulated. Previous cell encapsulation was performed using the spray-drying technique with the following wall materials: lactose-free milk, lactose-free milk and inulin, and lactose-free milk and oligofructose. Thus, three different probiotic powders were obtained and added separately to three fractions of concentrated lactose-free yogurt. The probiotic survival of both powders and yogurts was evaluated during refrigerated storage. Likewise, the viability of starter cultures in yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) was controlled. In addition, the physicochemical properties of the four yogurts were also measured (color, pH and acidity, and texture properties). All three powders showed good probiotic viability (>8 log CFU g^{-1}) throughout 120 days of storage at 4 ºC. In turn, yogurt formulations (with the addition of powders or free bifidobacteria) presented probiotic viability above 7 log CFU g^{-1} after storage; as well as the starter cultures (> 8 log UFC g^{-1}). Yogurt with probiotic powder from lactose-free milk showed a more yellowish color; however, these differences would not be detected by the human eye ($\Delta E < 3.00$). The yogurt with bifidobacteria free cells showed a greater post-acidification process (pH 4.18 to 4.02 and titratable acidity 1.52 to 1.89). It was not observed differences for firmness values of yogurt with free cells addition and yogurt with lactose-free milk and oligofructose powder addition. A slight significant decrease in the cohesiveness was observed in the yogurt elaborated with bifidobacteria free cells. The gumminess showed fluctuating values between all concentrated lactose-free yogurts. At the end of this study, we conclude that these probiotic powders can be incorporated into innovative lactose-free yogurts.

Keywords: Bifidobacteria, Lactose-free, Skyr-style yogurt, Probiotic food, Inulin, Texture.

1 INTRODUCTION

Probiotic microorganisms are known to provide several consumer health benefits when administered in adequate amounts. Strain characteristics and mechanisms of *Bifidobacterium* BB-12 against pathogen inhibition, barrier function enhancement, and immune interactions are mechanisms demonstrated for this type of bacteria. *Bifidobacterium* BB-12 has proven its beneficial health effect in numerous clinical studies within gastrointestinal health and immune function. Furthermore, bifidobacteria have been shown to improve bowel function, have a protective effect against diarrhea, and reduce side effects of antibiotic treatment, such as antibiotic-associated diarrhea. In terms of immune function, clinical studies have shown that bifidobacteria increases the body's resistance to common respiratory infections as well as reduces the incidence of acute respiratory tract infections microbiota (JUNGERSEN *et al*., 2014). Therefore, it is recommended that the minimum daily intake of probiotic viable cells should be between 10^6 and 10^7 CFU per g of food product (WANG; LIN; ZHONG, 2020).

Hansen, Allan-Wojtas, Jin, and Paulson (2002) highlighted that although bifidobacteria are being increasingly recognized as probiotics that have advantageous properties, they are also fastidious, obligate anaerobes, and, therefore, pose a technological challenge for the food industry. Therefore, the encapsulation of probiotic cells shows advantages and possibilities of incorporating produced particles into food matrices. Microencapsulation techniques, as spray drying, were used to achieve better probiotic survival during food processing, storage, and passage through the gastrointestinal tract (XAVIER DOS SANTOS *et al*., 2019; PINTO *et al*., 2015; VERRUCK *et al*., 2017). The spray drying technique enables the use of different types of encapsulating materials, among them, prebiotics and milk products have been employed for many types of research. Prebiotic like inulin or oligosaccharide is not metabolized by humans. The prebiotic property allows for unique applications such as determination of colonic targeting, making use of metabolization by microbiota present in the colon, or by probiotic bacterium present in the microparticles from the encapsulation process. Moreover, the presence of prebiotics in the composition of microparticles can result in a longer dissolution time in the water. According to Pinto *et al*. (2015), this long dissolution time ensures good control of the probiotic release when in contact with the dairy product water, such as of a concentrated yogurt.

Since lactose intolerance affects approximately 75% of the adult world population (SURI *et al*., 2019), products free of this disaccharide are essential to make up the diet of intolerant people. To avoid traces of lactose contamination in dairy products, lactose-free milk

and prebiotics were used by Dantas *et al*. (2021a) aiming for the future application of probiotic spray-dried powders in lactose-free dairy products. In this study, lactose-free skim milk was used as an innovative wall material for *Bifidobacterium* BB-12 encapsulation. Dantas *et al*. (2021a) observed better survival rates in spray-dried powders with encapsulated bifidobacteria (80 to 88%) than bifidobacteria-free cells (~75%), after in vitro gastrointestinal condition stages. These results encourage us to obtain an innovative dairy product.

A variety of dairy products has been elaborated with the addition of different probiotic microorganisms (ANGELOPOULOU *et al*., 2017; ASPRI *et al*., 2018; MACHADO *et al*., 2017; MEIRA *et al*., 2015; NADELMAN *et al*., 2017; RANADHEERA *et al*., 2016; SILVA *et al*., 2019; VERRUCK *et al*., 2020). Particularly, yogurt and other fermented milk products are the most common food carriers for probiotic bacteria (PINTO *et al*., 2019). Besides, concentrated fermented milks, for example, the skyr, has become popular in the past few years (KÖRZENDÖRFER *et al*., 2019). Skyr is a traditional product of Iceland made from skimmed sheep and cow milk, and fermented including a traditional starter (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) (MACORI; COTTER, 2018). The skyr has even gained popularity as a low-fat and high-protein food, being a very important dairy product of the inhabitants of Iceland, providing essential nutrients (STEINGRÍMSDÓTTIR; THORKELSSON; EYTHÓRSDÓTTIR, 2018). As above mentioned, lactose intolerance, a gastrointestinal disorder, can limit consumption of these types of food products. This gastrointestinal disorder is characterized by symptoms such as flatulence, diarrhea, abdominal pain, and bloating (ROŠKAR *et al*., 2017). Milk fermentation is a process that naturally reduces the lactose content (HARJU; KALLIOINEN; TOSSAVAINEN, 2012); nevertheless, *skyr*-style yogurt can contain significant intact amounts of this disaccharide. It is noteworthy that for a food product to be claimed as lactose-free, the concentration of lactose must be lower than 0.1 g 100 g^{-1}, according to the advice of the European Food Safety Agency (TRANI *et al*., 2017) and a new resolution in Brazilian legislation (BRASIL, 2017). Pereira *et al*. (2021) elaborated and evaluated a skyr yogurt with mango pulp, fructooligosaccharide, and natural sweeteners. The potential of lactose-free greek-style yogurt as a new matrix for incorporation of spray-dried microparticles containing the probiotic *Bifidobacterium* BB-12 was evaluated by Pinto *et al*. (2019). However, to our knowledge, the present study is the first report about lactose-free skyr-style yogurt.

Considering that different combinations of wall materials can result in spray-dried powders with different physical characteristics and structures, and based on the previous results obtained by Dantas *et al*. (2021a) and Dantas *et al*. (2021b), we noted the importance to evaluate

the impact of the application of these spray-dried powders on the lactose-free skyr-style yogurt. In this context, this study aimed to investigate the potential of spray drying encapsulation of *Bifidobacterium* BB-12 with lactose-free milk, lactose-free milk and inulin, and lactose-free milk and oligofructose in the production of a concentrated lactose-free yogurt. Therefore, the texture, physicochemical, and microbiological properties of the dairy product were assessed throughout 30 days of storage at 5 ºC. It was also evaluated the survival of the encapsulated probiotic bacteria during 120 days of storage under refrigeration temperature.

2 MATERIAL AND METHODS

2.1 MATERIALS

The probiotic *Bifidobacterium animalis* ssp. *lactis* BB-12 (NU-TRISH® BB-12®, Chr. Hansen, Hørsholm, Denmark) was employed as the active material for the microparticle. Lactose-free skim milk powder (Aurora®, Cooperativa Central Aurora Alimentos, Santa Catarina, Brazil) (85.51 g total solids 100 g^{-1}, 32.50 g protein 100 g^{-1}, 0.00 g fat 100 g^{-1}, 3.01 g ash 100 g^{-1} and 50.00 g carbohydrates 100 g^{-1}), inulin (DP ≥ 10, Orafti® Gr, Orafti, Tienen, Belgium) and oligofructose (2 ≤ DP ≤ 8, Orafti® P95, Orafti, Tienen, Belgium) were used as wall materials. Sodium propionate (Fluka, Neu-Ulm, Germany), lithium chloride (Vetec, Rio de Janeiro, Brazil), M17 agar (Sigma-Aldrich, St. Louis, MO, USA), AnaeroGen® (Oxoid, Hampshire, UK), MRS Agar (Merck, Darmstadt, Germany) and MRS Broth (Difco, Sparks, USA) were used for the microbiological assays. For the preparation of the concentrated lactose-free yogurt were used lactose-free skim milk (Aurora®, Cooperativa Central Aurora Alimentos, Santa Catarina, Brazil) (8.80 g total solids 100 mL^{-1}, 3.30 g protein 100 mL^{-1}, 0.32 g fat 100 mL^{-1}, 0.18 g ash 100 mL^{-1} and 5.00 g carbohydrates 100 mL^{-1}) and thermophilic starter culture containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Yo-Flex® L812, Chr. Hansen, Horrsholm, Denmark).

2.2 OBTAINING AND CHARACTERIZATION OF PROBIOTIC SPRAY-DRIED POWDERS

2.2.1 Encapsulation of *Bifidobacterium* BB-12

Three feed solutions were prepared for the production of spray-dried powders containing *Bifidobacterium* BB-12. The feed solutions prepared with sterile distilled water were obtained from lactose-free skim milk powder (200 g L^{-1}); lactose-free skim milk powder (100 g L^{-1}) and inulin (100 g L^{-1}); and lactose-free skim milk powder (100 g L^{-1}) and oligofructose (100 g L^{-1}), and denoted as 1, 2, and 3, respectively. The spray-dried powders derived from feed solutions 1, 2, and 3 were also denoted as 1, 2, and 3. A precipitate of probiotic cells was added to each feed solution. A laboratory-scale spray dryer (B 290 mini spray dryer, Buchi, Flawil, Switzerland) equipped with a cyclone was used to obtain the three spray-dried powders. The spray dried under optimum conditions used was 150 °C inlet temperature and 44 °C outlet temperature to obtain probiotic three spray-dried powders. The mini spray dryer, with an integrated standard two-fluid nozzle (0.7 mm liquid orifice diameter, a 1.1 mm liquid outer diameter, and a 1.5 mm gas orifice diameter), using compressed air, was used to disperse each one feed solutions into fine droplets. The compressor air pressure, drying airflow rate, and feed rate were set at 0.7 MPa, 35 m3 h^{-1}, and 12 mL min^{-1}, respectively. The powder and wet air were separated and collected by the cyclone. As observed by Dantas et al. (2021b), the three spray-dried powders formulation and operating conditions result in clusters formation, and therefore, the spray-dried powders contain microparticles, which can be called microspheres. However, it was chosen the denomination of microparticle in the present study.

2.2.2 Effect of storage on microparticle probiotic viabilityy

Spray-dried powders (1, 2, and 3) with bifidobacteria microparticles were stored for 120 days at refrigeration temperature (4 °C). Viable cells counts were performed every 30 days; for this, we used the following methodological steps: first, the entrapped bacteria were released from the microparticle according to Sheu, Marshall, and Heymann (1993) with some modifications. One gram of spray-dried powder was resuspended in 9 mL of sterile phosphate buffer (0.1 mol L^{-1}, pH = 7) followed by homogenization in a vortex (VTX-F-100; Biomixer, São Paulo, Brazil) for 10 min. Second, the mixtures were serially diluted in peptone water (Oxoid; 0.1 g 100 mL^{-1}), and inoculated on MRS agar modified by the addition of lithium chloride (0.2 g 100 g^{-1}) and sodium propionate (0.3 g 100 g^{-1}), as described by Vinderola and Reinheimer (1999). And finally, the plates were incubated at 37 °C for 72 h in anaerobic jars using AnaeroGen®. After the incubation period, the number of colonies formed on the agar was counted and expressed in log colony-forming units per gram (log CFU g^{-1}).

2.3 ELABORATION AND CHARACTERIZATION OF PROBIOTIC CONCENTRATED LACTOSE-FREE YOGURTS

2.3.1 Manufacture of probiotic concentrated lactose-free yogurts

Free *Bifidobacterium* BB-12 and the different spray-dried powders were incorporated into the concentrated lactose-free yogurt to produce novel dairy products. At the end of the fermentation process of the lactose-free skyr-style yogurt, each one of the spray-dried powders was incorporated individually for each formulation, in the amount of 10 g 100 g^{-1}. In turn, the suspension of free bifidobacteria (10 log CFU g^{-1}) was added in the amount of 10 mL 100 g^{-1}. The yogurt manufacture process began with the heating of the lactose-free skim milk until 42 °C. At this point, the milk was mixed with starter culture according to the producer's recommendation, fermented until 6 h and cooled to 18 ºC to stop the fermentation. The fermented milk was centrifuged (8000*g*) (Nova Técnica NT825, São Paulo, Brazil) at this same temperature for 15 min, as suggested by Moineau-Jean *et al*. (2019). The whey was discarded and the concentrated curds were pooled. Then, the incorporation of the additives was performed, giving rise to the distinct probiotic concentrated lactose-free yogurts, which are presented in Table 9. Finally, the yogurts were transferred to a refrigerator (5 °C ± 1) and stored during thirty days. The microbiology of the product and its chemical and physical properties were studied at storage days 0, 15 and 30.

Table 9 − Description of the different types of probiotic concentrated lactose-free yogurt.

Product	Definition
Yogurt FC	Concentrated lactose-free yogurt added of free *Bifidobacterium* BB-12
Yogurt SDP1	Concentrated lactose-free yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free milk powder
Yogurt SDP2	Concentrated lactose-free yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free milk powder and inulin
Yogurt SDP3	Concentrated lactose-free yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free milk powder and oligofructose

2.3.2 Microbiological analysis

Twenty-five grams of each yogurt were diluted in 225 mL of phosphate buffer (pH 7.0, 0.1 mol L^{-1}) followed by homogenization using a magnetic stirrer for 10 min. Serial dilutions were made according to the methodology by Vinderola and Reinheimer (1999) already described (section 2.2.2). So, plates incubation and enumeration of *Bifidobacterium* BB-12 also were performed according to section 2.2.2.

The *S. thermophilus* count was carried out by the pour plate technique using M17 agar with addition of lactose solution (10 g 100 mL^{-1}), incubated aerobically at 37 ± 1°C for 48 h (IDF, 1997). Enumeration of *L. delbrueckii* subsp. *bulgaricus* was realized using MRS agar under aerobic conditions at 37 ± 1 °C for 72 h (DAVE; SHAH, 1996). The total viable count (determined in triplicate) was expressed as log of colony-forming unit per gram of yogurt (log CFU g^{-1}).

2.3.3 Physical and chemical properties

The textural properties of the yogurts were measured using a texturometer model TA-HD plus (Stable Micro System, Texture Analyser, Surrey, UK). The double compression analysis was carried out using a 25 mm-diameter aluminum probe (P25/L). The analysis was performed in a 50 mL glass capsule with the samples at temperature of 6 ± 1 ºC. The test velocity, the time, and the distance were set at 1.0 mm s^{-1}, 5.0 s, and 10.0 mm, respectively. The parameters firmness, gumminess, and cohesiveness were obtained by the software Exponent version 6.1.1.0. (Stable Micro Systems, Surrey, United Kingdom).

A pH meter (model PHS3BW, BEL Engineering, Monza, Italy) was employed to measure the pH values, while the titratable acidity (% lactic acid) was determined in accordance to AOAC (2005).

Color measurements were carried out using a Chromameter CR-400 (Konica Minolta, Osaka, Japan) with illuminant D65. The results were expressed in accordance to CIELab coordinate colour space system; that is, the L^* component (lightness, it ranges from black to white), and the a^* (+, red; −, green) and b^* (+, yellow; −, blue) parameters were determined. Hue angle (h) and Chroma (C^*) values also were presented. The total color difference (ΔE*), between values observed in the final storage time (day 30) with initial time (day 0) of each concentrated yogurt was calculated, as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

where ΔL* is the difference of luminosity, Δa* is the difference of the parameter a*, and Δb* represents the difference of the parameter b*, for the same sample.

2.4 STATISTICS

The probiotic concentrated lactose-free yogurts were manufactured through the three experimental trials and were carried out in independent days. All samples analyses were done in triplicate. To evaluate significant differences ($P < 0.05$) between treatments, one-way analysis of variance (ANOVA) and the t-test were used. The software STATISTICA version 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) was employed for all statistical analyses, and the results were expressed as mean ± standard deviation.

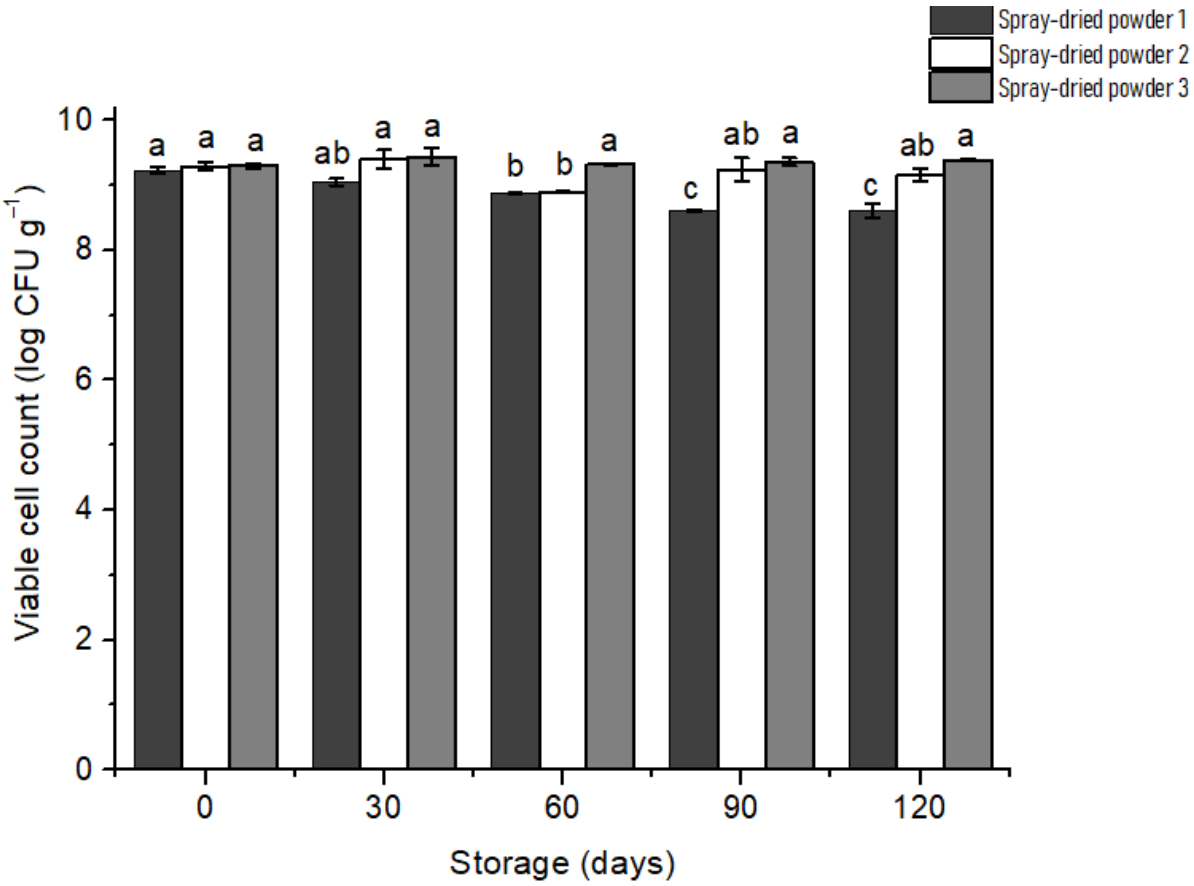
3 RESULTS AND DISCUSSION

3.1 SURVIVAL OF MICROENCAPSULATED *BIFIDOBACTERIUM* BB-12 DURING STORAGE

On the day of obtaining of the spray-dried powders, all samples showed viable cells count higher than 9.00 log CFU g^{-1}, which characterize them as a probiotic product. The characterization and the monitoring of the microparticle during storage at different temperatures are also important approaches to improve and determine the optimal conditions when adding them to food products (VERRUCK *et al*., 2018).

The viable *Bifidobacterium* BB-12 cell counts throughout the storage time of 120 days at 4 ± 1°C are shown in Figure 9. Our findings suggest that lactose-free milk powder or its partial replacement with inulin or oligofructose in the spray drying process caused no negative effect on the bacteria survival. Verruck et al. (2018), who also studied the refrigerated storage of probiotic powders, did not observe a reduction in the viable cell count of *Bifidobacterium* BB-12 in a spray-dried powder based on full-fat goat's milk and inulin or oligofructose. However, in the present study the greatest change was found for the spray-dried powder 1, in which there was a decrease in viability over time, varying from 1.95–6.83%, but like the others, remained within the probiotic pattern (equal to or greater than 6 log CFU g^{-1} of the product). As documented by our previous study (DANTAS *et al*., 2021), spray-dried powder 1 presented the largest initial moisture and a_w (7.67 g 100 g^{-1} and 0.396, respectively). Liu *et al*. (2016) stated that as a higher the moisture content, the lower the bacterial survival during storage, and that therefore, an ideal moisture content would be around 4 g 100 g^{-1}. Himmetagaoglu and Erbay (2019) highlighted that in a water activity lower than 0.3, a better shelf-stability is achieved, since microbiological growth and chemical reactivity (such as non-enzymatic browning) are at a minimum. Nevertheless, the good results for all samples may be justified by the fact that the bacterial cells are in latent state in the conditions of storage at 4°C, and consequently the rates of chemical reaction are reduced (PEDROSO *et al*., 2012). Muhammad *et al*. (2017) also affirmed that storage temperature and residual moisture content are crucial factors for the increase or decrease of the probiotic viability by influencing the lipid oxidation of membrane. Finally, our results are in agreement with those found by Rodriguez-Restrepo, Giraldo, and Rodriguez-Barona (2017), who studied the viability of *Bifidobacterium animalis* subsp. *lactis* encapsulated by spray drying with gum arabic and whole milk powder, after 140 days of storage at 6 ± 2 °C.

Figure 9 − Viable *Bifidobacterium* BB-12 cells count from spray-dried powders during 120 days of storage at 4 ± 1 °C.



[a, b, c]Different letters in the top denote significant differences ($P < 0.05$) for a same sample during the storage period.

3.2 CHARACTERIZATION OF THE PROBIOTIC CONCENTRATED LACTOSE-FREE YOGURTS

3.2.1 Viability of the probiotic and starter cultures

For all the formulations of yogurt, the viable cell count of *Bifidobacterium* BB-12 was more than 7 log CFU g^{-1}, as recommended to get healthy benefits (Table 10). Abd El-Salam *et al*. (2011) affirmed that the high viability of probiotics-free cells added in concentrated fermented milk (in their case, labneh) may be related to the high total solids content. They noted that the counts of *L. casei* and *L. acidophilus* remained above 8 log CFU g^{-1} throughout the storage period. According to Iravani, Korbekandi, and Mirmohammadi (2015), one of the

factors that restrict the stability of probiotic bacteria-free cells in fermented products is the low pH. It was observed a decrease of the pH values, and at the same time, a slight decrease ($P < 0.05$) in the viability of *Bifidobacterium* BB-12 free cells, however, this not affected the probiotic properties of concentrated lactose-free yogurt. Similar behavior was observed by Pinto *et al*. (2019), who investigated the incorporation of *Bifidobacterium* BB-12 free cells and encapsulated in Greek-style yogurt.

In the previous study, published by Dantas *et al*. (2021a), the focus was on the shelf-life stability of bifidobacteria using the same formulation of spray-dried powders employed in the present work. Moreover, the authors studied bifidobacteria's *in vitro* gastrointestinal survival, which is a more accurate predictor of performances studies for probiotic bacterial survival. This study also confirmed that bifidobacteria free cells cannot be replaced by the spray-dried powders because after the under *in vitro* simulated gastrointestinal conditions assay, the free cells survival rate was minor than any other spray-dried powder (1, 2, or 3) formulated, prepared again, and used in the present study. Survival rates (%) of *Bifidobacterium* BB-12 after under simulated gastrointestinal for free cells and spray-dried powders 1, 2 and 3 were equals to 75.53%, 80.01%, 81.29%, and 87.59%, respectively (DANTAS *et al*., 2021a). In turn, results presented by Dantas *et al*. (2021b) were necessary to understand the behavior of the wall materials employed on the survival of *Bifidobacterium* BB-12. If this study did not demonstrate good results, there would be no reason to apply these spray-dried powders formulations in skyr-style yogurt or any other product. Therefore, it was detected previously the additional protection guarantee of *Bifidobacterium* BB-12 microencapsulation process, using feed solutions with lactose-free skim milk powder, lactose-free skim milk powder and inulin, and lactose-free skim milk powder and oligofructose. Based on these previous results, it was possible to conclude that the potential protective effect of encapsulation occurs during the passage of the bifidobacteria through the gastrointestinal tract, and it is not only represented by survival rates (%) during 30 days of storage observed in the present study. These survival rates (%) were equals to 92.54%, 90.91%, 87.84%, and 86.03%, for yogurt added of *Bifidobacterium* BB-12 free cells, yogurt added of spray-dried powder 1, yogurt added of spray-dried powder 3, and yogurt added of spray-dried powder 2, respectively.

Table 10 – Viable cells count of *B. lactis* BB-12 and starter cultures in the yogurts.

		Number of viable cells (log CFU g^{-1})		
Product	Day	*Bifidobacterium* BB-12	*Streptococcus thermophilus*	*Lactobacillus bulgaricus*
Yogurt FC	0	8.887 ± 0.130^{Aa}	9.261 ± 0.032^{Aa}	8.328 ± 0.016^{Ba}
	15	8.498 ± 0.031^{Ab}	9.175 ± 0.010^{Bab}	8.392 ± 0.160^{Ba}
	30	8.224 ± 0.278^{Ab}	8.987 ± 0.088^{ABb}	8.447 ± 0.022^{ABa}
Yogurt SDP1	0	8.866 ± 0.180^{Aa}	9.357 ± 0.001^{Ab}	8.335 ± 0.013^{Bb}
	15	8.504 ± 0.159^{Ab}	9.460 ± 0.003^{Aa}	8.618 ± 0.007^{ABa}
	30	8.060 ± 0.145^{Ac}	9.166 ± 0.023^{Ac}	8.572 ± 0.057^{Aa}
Yogurt SDP2	0	9.123 ± 0.092^{Aa}	9.379 ± 0.084^{Aa}	8.557 ± 0.001^{Aa}
	15	8.553 ± 0.087^{Ab}	9.629 ± 0.005^{Aa}	8.597 ± 0.008^{ABa}
	30	7.849 ± 0.298^{Ac}	8.786 ± 0.075^{Bb}	7.910 ± 0.128^{Bb}
Yogurt SDP3	0	8.975 ± 0.087^{Aa}	9.274 ± 0.003^{Aab}	8.411 ± 0.073^{Ba}
	15	8.731 ± 0.164^{Aa}	9.534 ± 0.131^{Aa}	8.703 ± 0.018^{Aa}
	30	7.884 ± 0.092^{Ab}	8.998 ± 0.022^{ABb}	8.248 ± 0.211^{ABa}

[A,B,C,D]Within a column, different superscript uppercase letters denote significant differences ($P < 0.05$) among the different yogurts for the same storage period.
[a,b,c]Within a column, different superscript lowercase letters denote significant differences ($P < 0.05$) among the different storage day, for each sample.
Yogurt FC: yogurt added of free *Bifidobacterium* BB-12; Yogurt SDP1: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced only with lactose-free milk powder; Yogurt SDP2: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free milk powder and inulin; Yogurt SDP3: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 microcapsules produced with lactose-free milk powder and oligofructose.

Viable *S. thermophilus* count showed high values and slight variations ($P < 0.05$) during storage time (Table 10). Dimitrellou *et al*. (2016), Pinto *et al*. (2019) and Varga, Sule, and Nagy (2014) reported analogous results for yogurt or similar fermented milk products stored under refrigeration for 4–6 weeks. *S. thermophilus* generally survives well (>10^8 CFU mL^{-1}) in these products. Moreover, De Souza Oliveira *et al*. (2011) observed reduction in the generation time (t_g) for *S. thermophilus* and *L. bulgaricus* when inulin was used in skim milk fermented, suggesting an effect of the prebiotic on these pure cultures, and not just for the other bacteria (probiotic strains) used in the study. In this work, the viability of *L. bulgaricus* was higher ($P < 0.05$) on day of manufacture in the yogurt added with spray-dried powder 2

(microcapsule from milk and inulin) compared with the other yogurts. In summary, our findings showed satisfactory values of viability for starter cultures in all yogurt samples, since they were greater than 7 log CFU g^{-1}, and thus are in accordance with the quality parameters established by Brazilian legislation (BRASIL, 2007) for fermented milks.

3.2.2 Color measurements

Table 11 shows the color parameters for the yogurts during the storage period. Overall, it was observed that the L^* values (lightness) were not different ($P > 0.05$) among the yogurts with spray-dried powders addition on day 1, being that all samples displayed high lightness values. However, for yogurt with free cells of bifidobacteria was observed a decrease in lightness values on day 15, followed by an increase on day 30. Similar results were verified by De Campo *et al*. (2019), who incorporated zeaxanthin nanoparticles in yogurt. Regarding L^* values, these authors observed a decrease on day 7 following by an increase on day 28. De Campo *et al*. (2019) related that the slight decrease in L^* values can be related to the casein present in milk. When used free cells, the protein interacts with the protease of the bacterial culture, and the proteolysis reaction can reduce the L^* intensity (De CAMPO *et al*., 2019). Moreover, these authors also affirmed that fat globules and casein micelles contribute to the white appearance due to their capacity to scatter light, which also can explain the increase in L^* values on day 30 in the present study.

It was possible to verify a variation ($P < 0.05$) in the yellow coloration (b^* values) among the yogurts. Verruck, Dantas, and Prudencio (2019) discussed that the yellowish coloration is natural in dairy products prepared with cow's milk because cows transfer high carotenoid levels from their diet to milk. Since Yogurt SDP1 was added to the probiotic spray-dried powder made exclusively from milk, this explains its more yellowish color ($P < 0.05$) compared to the other samples. Concerning the a^* parameter, despite significant differences ($P < 0.05$) between all samples for any storage day, it was observed a general tendency to greenish coloration, since all showed negative values. Canella *et al*. (2018) commented that this coloration may be attributed to the content of riboflavin naturally present in the milk. Furthermore, our results agree with those noted by Debon *et al*. (2012), who studied color measurements of prebiotic fermented milk during 28 days of storage.

Table 11 − Color parameters of the yogurt with free probiotic and yogurts added of spray-dried powders during 30 days of storage at 5 °C.

	Day	Yogurt FC	Yogurt SDP1	Yogurt SDP2	Yogurt SDP3
*L**	0	93.85 ± 0.89^{Ab}	93.32 ± 0.48^{Aa}	94.50 ± 0.82^{Aa}	93.47 ± 1.07^{Aa}
	15	79.70 ± 0.96^{Cc}	91.60 ± 0.65^{Bb}	93.85 ± 0.45^{Aa}	93.29 ± 0.17^{Aa}
	30	95.49 ± 0.33^{Aa}	92.41 ± 0.78^{Bab}	93.43 ± 0.51^{Ba}	93.10 ± 0.60^{Ba}
*a**	0	-2.06 ± 0.01^{Dc}	-1.27 ± 0.01^{Ab}	-1.69 ± 0.03^{Cac}	-1.56 ± 0.02^{Bb}
	15	-1.41 ± 0.08^{Ca}	-1.08 ± 0.01^{Aa}	-1.21 ± 0.09^{Ba}	-1.19 ± 0.02^{ABa}
	30	-1.91 ± 0.01^{Db}	-1.10 ± 0.06^{Aa}	-1.37 ± 0.02^{Cb}	-1.19 ± 0.02^{Ba}
*b**	0	8.39 ± 0.15^{Cb}	10.57 ± 0.04^{Ac}	9.74 ± 0.04^{Bb}	9.80 ± 0.11^{Bb}
	15	10.68 ± 0.30^{Ba}	11.20 ± 0.06^{Ab}	10.28 ± 0.31^{Ba}	9.72 ± 0.10^{Cb}
	30	8.21 ± 0.18^{Db}	11.62 ± 0.07^{Aa}	9.82 ± 0.02^{Cb}	10.12 ± 0.10^{Ba}
*C**	0	8.64 ± 0.15^{Cb}	10.65 ± 0.04^{Ac}	9.89 ± 0.04^{Ba}	9.92 ± 0.12^{Bb}
	15	10.62 ± 0.29^{Ba}	11.25 ± 0.06^{Ab}	10.19 ± 0.30^{BCa}	9.79 ± 0.11^{Cb}
	30	8.40 ± 0.21^{Db}	11.68 ± 0.07^{Aa}	9.92 ± 0.02^{Ca}	10.19 ± 0.10^{Ba}
h	0	103.77 ± 0.16^{Aa}	96.86 ± 0.07^{Ca}	99.50 ± 0.54^{Ba}	98.89 ± 0.13^{Ba}
	15	97.15 ± 0.80^{Ab}	95.45 ± 0.12^{Bb}	97.10 ± 0.68^{Ab}	96.94 ± 0.13^{Ab}
	30	103.14 ± 0.38^{Aa}	95.88 ± 0.47^{Db}	97.92 ± 0.12^{Bb}	96.70 ± 0.16^{Cb}
ΔE*		1.68	1.40	1.12	0.61

[A,B,C,D]Within a line, different superscript uppercase letters denote significant differences ($P < 0.05$) among the different yogurts for the same storage period.
[a,b,c]Within a column, different superscript lowercase letters denote significant differences ($P < 0.05$) among the different storage days, for each sample.
*L**: lightness value, it defines black at 0 and white at 100; *a**: it is relative to the green–red opponent colors, with negative values toward green and positive values toward red; *b**: it represents the blue–yellow opponents, with negative numbers toward blue and positive toward yellow; *C**: polar coordinate (chroma, relative saturation); *h*: polar coordinate (hue angle); ΔE*: the total color difference observed between the final storage time (day 30) and initial time (day 0).

Since the Hue angle shows the location of the color in a diagram (where 0°, 90°, 180°, and 270° represent pure red, pure yellow, pure green, and pure blue, respectively) (JHA, 2010), the values obtained for this parameter corroborate with the values of *a** and *b**, as they indicate that all yogurts showed a tendency towards slightly greenish-yellow. Despite these similarities, yogurt SDP1 showed the lowest *h* value among all samples, during the entire storage period, reinforcing the fact that it is more yellow than the others yogurt samples. Although of these differences, it is important to highlight that all samples of concentrated lactose-free yogurt

showed low ΔE* values. According to Martínez-Cervera *et al*. (2011), it is expected a ΔE* result ≤ 3, so the human eye does not notice that color differences.

Sołowiej et al. (2015) reported that C* represents color saturation, i.e., it is the combination of the parameters a* and b*, which shows the proportions in which the color is mixed with white, black, or gray. Overall, an increase of *C** values was observed during storage. According to Rozycki *et al*. (2010), this phenomenon is related to the accumulation of oxidation products that can react with amino groups forming yellow products. Canella *et al*. (2018) also verified an increase of the parameter *C** after 30 days of storage in a symbiotic fermented lactic beverage.

3.2.3 pH, acidity and texture analyses

As disclosed in Table 12, the pH values decreased ($P < 0.05$) for all yogurt formulations during the storage period. The yogurt FC showed lower pH values ($P < 0.05$) than the other yogurts during the entire storage period. Pinto *et al*. (2017) and Zomorodi (2019) also reported lower pH values in yogurts containing free bifidobacteria in comparison with their microencapsulated forms. The inverse pattern was observed for titratable acidity. This behavior is called post-acidification, and according to Pinto *et al*. (2019), it probably results from the residual activity of the starter cultures. It is well known that *L. bulgaricus* and *S. thermophilus* ferment glucose. Ribeiro *et al*. (2014) discussed that post-acidification is undesirable for both the microbial viability and sensory quality of yogurt. According to Pan, Liu, Luo, and Luo (2019), this phenomenon results in the rupture of casein strands, size decrease of casein micelles aggregates, and protein rearrangement in the yogurt gel, this leads to its shrinkage and consequent whey separation, i.e., syneresis.

Table 13 shows the evolution of texture parameters (firmness, gumminess, and cohesiveness) during refrigerated storage of probiotic concentrated lactose-free yogurts. De Campo *et al*. (2019) and Kesenkaş *et al*. (2017) also observed similar changes in the texture parameters during cold storage of yogurts. However, the addition of spray-dried powders 1 and 2 contributed to an increase ($P < 0.05$) in the firmness of the yogurt. The incorporation of powders tends to increase the total solids content, which leads to an increase in the firmness values. These results corroborate those obtained by Karaca, Saydam, and Guven (2019), since the addition of persimmon and apple powders significantly affected the instrumental firmness of low-fat and fat-free probiotic yogurts. In the Yogurt SDP2, the presence of inulin in the spray-dried powder 2 probably contributed to the increase of firmness parameter. Gyawali and

Ibrahim (2016) highlighted that inulin is a good stabilizer because it immobilizes the water molecules in the food matrix, contributing to the increase of the firmness parameter. Costa *et al.* (2019) noted that samples of Greek yogurt with inulin addition presented higher firmness values due to the increase in gel strength.

Table 12 – pH and titratable acidity of the concentrated lactose-free yogurts containing free or microencapsulated *B. lactis* BB-12 during storage.

Product	Day	pH	Titratable acidity (g/100 g)
Yogurt FC	0	4.18 ± 0.04^{Ca}	1.52 ± 0.01^{Ab}
	15	4.10 ± 0.02^{Cb}	1.62 ± 0.02^{Ab}
	30	4.02 ± 0.01^{Cc}	1.89 ± 0.07^{Aa}
Yogurt SDP1	0	5.06 ± 0.01^{Aa}	1.44 ± 0.01^{Ba}
	15	4.89 ± 0.05^{Ab}	1.52 ± 0.04^{Ba}
	30	4.47 ± 0.02^{Ac}	1.44 ± 0.04^{Ba}
Yogurt SDP2	0	4.79 ± 0.01^{ABa}	1.46 ± 0.03^{ABa}
	15	4.60 ± 0.02^{Bb}	1.49 ± 0.01^{Ba}
	30	4.41 ± 0.03^{Bc}	1.49 ± 0.02^{Ba}
Yogurt SDP3	0	4.68 ± 0.18^{Ba}	1.48 ± 0.02^{ABa}
	15	4.60 ± 0.03^{Bab}	1.48 ± 0.01^{Ba}
	30	4.39 ± 0.01^{Bb}	1.50 ± 0.04^{Ba}

[A,B,C]Within a column, different superscript uppercase letters denote significant differences ($P < 0.05$) among the different yogurts for the same storage period.
[a,b,c]Within a column, different superscript lowercase letters denote significant differences ($P < 0.05$) among the different storage day, for each sample.
Yogurt FC: yogurt added of free *Bifidobacterium* BB-12; Yogurt SDP1: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 produced only with lactose-free milk powder; Yogurt SDP2: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 produced with lactose-free milk powder and inulin; Yogurt SDP3: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 produced with lactose-free milk powder and oligofructose.

Table 13 − Textural properties of the concentrated lactose-free yogurts.

Product	Day	Firmness (g)	Gumminess (g)	Cohesiveness
Yogurt FC	1	55.61 ± 1.14^{Cb}	36.30 ± 1.23^{Cb}	0.653 ± 0.009^{Ba}
	15	44.50 ± 1.87^{Bc}	28.07 ± 1.18^{Bc}	0.631 ± 0.000^{ABa}
	30	71.85 ± 1.95^{Ba}	42.52 ± 0.86^{Ca}	0.592 ± 0.004^{Bb}
Yogurt SDP1	1	85.49 ± 2.36^{Aab}	51.66 ± 1.11^{Aa}	0.715 ± 0.021^{Aa}
	15	71.79 ± 13.43^{ABb}	62.23 ± 10.62^{Aa}	0.649 ± 0.013^{Aa}
	30	119.52 ± 6.02^{Aa}	72.17 ± 0.31^{ABa}	0.651 ± 0.033^{ABa}
Yogurt SDP2	1	62.98 ± 1.14^{Bb}	51.66 ± 0.99^{Bc}	0.683 ± 0.004^{ABa}
	15	95.68 ± 1.95^{Aab}	56.54 ± 1.63^{Ab}	0.610 ± 0.004^{Ba}
	30	129.42 ± 15.80^{Aa}	78.91 ± 2.63^{Aa}	0.614 ± 0.054^{ABa}
Yogurt SDP3	1	54.58 ± 0.98^{Cc}	37.43 ± 0.01^{Cb}	0.671 ± 0.008^{ABb}
	15	76.11 ± 2.60^{Ab}	49.45 ± 2.61^{ABab}	0.641 ± 0.000^{Ab}
	30	94.70 ± 6.76^{ABa}	60.42 ± 5.71^{Ba}	0.726 ± 0.013^{Aa}

^{A,B,C}Within a column, different superscript uppercase letters denote significant differences ($P < 0.05$) among the different yogurts for the same storage period.
^{a,b,c}Within a column, different superscript lowercase letters denote significant differences ($P < 0.05$) among the different storage day, for each sample.
Yogurt FC: yogurt added of free *Bifidobacterium* BB-12; Yogurt SDP1: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 produced only with lactose-free milk powder; Yogurt SDP2: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 produced with lactose-free milk powder and inulin; Yogurt SDP3: yogurt added of spray-dried powder which contain *Bifidobacterium* BB-12 produced with lactose-free milk powder and oligofructose.

The yogurt FC and the yogurt SDP3 not show differences ($P > 0.05$) between the firmness values on day 1. Costa *et al*. (2019) found similar results for Greek yogurt, a type of concentrated yogurt, and highlighted that the oligofructose was not able to influence the firmness values. This is because the presence of this prebiotic reduced the interaction forces of the protein matrix when compared to the addition of other prebiotics like inulin, polydextrose, and galactooligosaccharide. Furthermore, they cited that the smaller particle size of the oligofructose (73 μm and 89 μm for oligofructose and inulin, respectively) results in a lubricating effect, contributing to the non-increase of firmness value. As the gumminess is the product of the firmness and the cohesiveness, the gumminess showed fluctuating values between the values obtained for both parameters (firmness and cohesiveness).

A slight decrease ($P < 0.05$) in the cohesiveness was observed in the Yogurt FC during the storage period. Pinto *et al*. (2017) discussed that cohesiveness is a measurement of how well a product resists a second deformation relative to its resistance under the first deformation. It can be interpreted as how firm the linkage inside the gel needs to be to withstand deformation. Accordingly, a diminution in cohesiveness value could denote a lower association between protein-protein bindings after 15 days of storage. Bedani *et al*. (2014) and Pinto *et al*. (2019) also reported similar results during storage of probiotic soy yogurts added with inulin and/or okara flour, and for lactose-free Greek yogurts with free or encapsulated cells, respectively.

4 CONCLUSIONS

Overall satisfactory viability of *Bifidobacterium* BB-12 was found for all spray-dried powders produced with lactose-free skim milk powder, lactose-free skim milk powder and inulin, and lactose-free skim milk powder and oligofructose, when stored under refrigerated conditions for 120 days. Concerning the yogurts, the viability of *S. thermophilus* and *L. bulgaricus* was not critically affected during storage of the samples. Bifidobacteria survival rates (%) during 30 days increased as follows: yogurt with free cells > yogurt with spray-dried powder 1 > yogurt with spray-dried powder 3 > yogurt with spray-dried powder 2. Despite that, for all the formulations of yogurt, the viable cell count of *Bifidobacterium* BB-12 was higher than recommended to exert health benefits. Therefore, we continue to conclude that bifidobacteria free cells cannot be replaced by these spray-dried powders formulations, because after the *in vitro* simulated gastrointestinal conditions previously realized, the free cells survival rate was minor than of these spray-dried powders. Thus, given the higher post-acidification observed for yogurt added of free cells, we conclude that the spray-dried powders are the best choices for developing a probiotic lactose-free skyr-style yogurt.

Declaration of competing interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

The authors are grateful to Coordination of Improvement of Higher Education Personnel (CAPES, Brazil) [CAPES-PRINT, Project number 88887.310560/2018-00]; to National Council for Scientific and Technological Development (CNPq, Brazil) by the financial support [CNPq, 405965/2016-8], and to Coordination of Improvement of Higher Education Personnel (CAPES, Brazil) by the scholarship [001].

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CAPÍTULO 5

The combined use of progressive and block freeze concentration in lactose-free milk: effect of process parameters and influence on the content of carbohydrates and proteins

*** Trabalho publicado (redação na íntegra)**

Received: 30 May 2021 | Revised: 19 July 2021 | Accepted: 17 August 2021

DOI: 10.1111/jfpe.13867

ORIGINAL ARTICLE

Journal of Food Process Engineering

WILEY

The combined use of progressive and block freeze concentration in lactose-free milk: Effect of process parameters and influence on the content of carbohydrates and proteins

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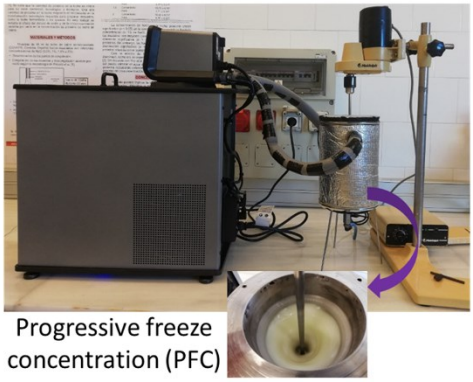
The combined use of progressive and block freeze concentration in lactose-free milk: effect of process parameters and influence on the content of carbohydrates and proteins

ABSTRACT

This work focuses on the study of the concentration of lactose-free milk using a combination of progressive freezing concentration (PFC) with block freezing concentration (BFC). First, we investigated the PFC of skim lactose-free milk applying Response Surface Methodology. To analyze the influence of three factors (stirring rate, coolant temperature, and assay time) on the response variables (concentrate yield, efficiency of the process, concentration index and average ice growth rate), a central composite design was used previously. It was found that all factors had a significate influence on the responses. Then, once the optimized condition for this step was chosen (time of 58 min, coolant temperature of – 5 ºC, and mechanical stirring of 1035 rpm), the ice obtained from it was subjected to a new freeze concentration cycle using the BFC assisted by vacuum. In the concentrated fraction of this cycle, protein and carbohydrate contents were equals to 6.7 g 100 g^{-1} and 10.24 g 100 g^{-1}, respectively. The results suggest that in PFC carbohydrates accumulate more in the concentrated phase, while in BFC it is the protein that has the greatest tendency to pass into the concentrated liquid. In this approach, we believe that it is possible to combine the two techniques (PFC + vacuum-assisted BFC) to obtain concentrates, and that they can be used for the development of innovative lactose-free dairy products.

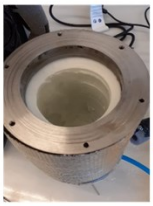
Keywords: Concentrate, Lactose-free dairy, Protein, Skim milk, Vacuum freeze concentration

Lactose-free milk
TS: 8.8 % w/w



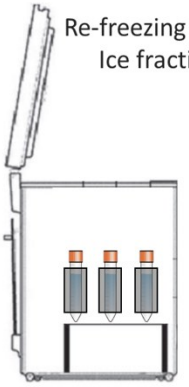
Progressive freeze concentration (PFC)

TS: 11.58 % w/w
Concentrated fraction

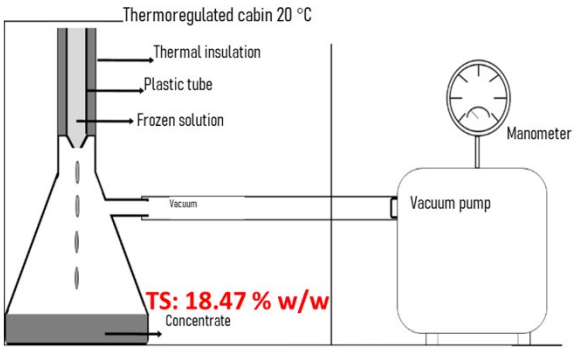


Ice fraction
TS: 4.61 % w/w

Melting of the Ice fraction



Re-freezing of the Ice fraction



Block freeze concentration
(with the Ice fraction obtained from PFC)

	CI	Y (%)
PFC	1.33	80
BFC	4	80
GLOBAL	1.42	97

TS = TOTAL SOLIDS

PRACTICAL APPLICATIONS

The use of concentration processes can facilitate the customization of milk products rich in proteins to meet specific requirements on nutritional and functional properties, for instance in bakery products, formulated food, ice-cream, beverages, energy drinks, and others. Moreover, since most changes occur in an aqueous environment, the removal of some part of milk water results in its preservation. Within the concentration processes, the freeze concentration emerges swiftly thanks to its inherent features, involving low-temperature processing and selective nature of the water removal step. Because of the low temperatures used in freeze concentration, this technology is gaining in popularity as an alternative technique to the standard concentration techniques currently used in dairy processing. It offers the most enhanced functional and sensorial qualities of concentrated milk because it decreases the quality deviation by minimizing the heat abuse on sensitive milk components, such as proteins, water-soluble vitamins, and aromatics.

1 INTRODUCTION

The incidence of lactose intolerance varies according to the regions of the world. Ugidos-Rodríguez, Matallana-González, and Sánchez-Mata (2018) reported that the occurrence of this gastrointestinal disorder is approximately 70% in the adult world population and 90% in the Asian population. In the case of the African and South American populations, it is estimated that 50% of people are affected (NEVES; DE OLIVEIRA, 2021). The disaccharide lactose is composed of one unit of galactose and one of glucose linked by β-1, 4 glycosidic bonds. In the human body, this linkage is naturally hydrolyzed by a specific enzyme, β-galactosidase (SINGH *et al*. 2021). So, a deficient amount of this enzyme or its complete absence leads to malabsorption of lactose; which in turn is associated with symptoms such as abdominal pain and bloating, flatulence, diarrhea, and vomiting (SURI *et al*., 2019). Therefore, when considering this whole scenario, it is necessary to develop alternative and innovative technologies to serve lactose intolerants who do not want to restrict dairy products from their diet, given the importance of specific nutrients present in milk.

Freeze concentration is already a widespread and accepted technology, applied mainly in the elaboration of ingredients and foods that have high nutritive value (AIDER; HALLEUX, 2009). In this process, the concentration of the fluid occurs through the removal of water in the form of ice crystals. This means that the temperature of food liquid decreases below its freezing point (GULFO *et al*., 2014), aiming to avoid the eutectic temperature and consequently the solidification of all product constituents. One of the main advantages of this operation is the preservation of thermally sensitive compounds, given the low processing temperatures employed (DE LIZ *et al*., 2020). The milk treatment in a temperature range from 70 to 100°C can denature the whey protein (e.g., α-lactalbumin and β-lactoglobulin) and induce the formation of aggregates (QIAN *et al*., 2017). When comparing heat of evaporation of water (about 2260 kJ kg^{-1} under at pressure of 0.1 MPa) with the enthalpy of freezing water (335 kJ kg^{-1}), the process of freeze concentration seems to be cheaper than evaporation from the energy point of view. However, in practice, multi-effect evaporation is available to recover thermal energy, causing the difference in energy requirement between the two concentration methods to be substantially reduced (MIYAWAKI *et al*., 2005).

Among freeze concentration processes, progressive freeze concentration (PFC) has been studied to concentrate various liquid foods (AZHAR *et al*., 2020; ROSDI *et al*., 2020; MIYAWAKI *et al*., 2017), as well as effluents (MOHAMED ANUAR; AMRAN; RUSLAN, 2020; MAZLI *et al*., 2021). This technique consists of the partial freezing of the solution under

constant agitation, which is generally employed to decrease the solute retention in the ice sheet (OJEDA *et al*., 2017). This ice sheet is formed on a cooled surface (tank walls) and is then separated from the concentrated fluid when the operation is finished. On the other hand, in the block freeze concentration (BFC), the food liquid to be concentrated is completely frozen, followed by partial gravitational thawing. Therefore, the ice block represents a solid carcass through which the concentrated liquid passes (MACHADO CANELLA *et al*., 2018). Aiming to enhance separation efficiency, the BFC can be assisted by other techniques, such as the vacuum (ORELLANA-PALMA *et al*., 2017) and centrifugation (BAYKAL; DIRIM, 2019).

The combined use of different concentration techniques has also been reported in the literature; for example, suspension freeze concentration and centrifugal filtration of apple juice (QIN *et al*., 2021), and PFC of coconut water followed by controlled thawing of ice (JAYAWARDENA; VANNIARACHCHY; WANSAPALA, 2020). Recent work by Hernández *et al*. (2021) suggests the combination of PFC and BFC assisted by vacuum, as a strategy for desalination of saline solutions simulating seawater. On the other hand, no references have been found on the application of freeze concentration to lactose-free milk.

The approach of this study provides first data on the use of freeze concentration processes to concentrate nutrients and compounds from lactose-free milk. Therefore, response surface methodology (RSM) was used to determine the optimum parameter conditions for the PFC of milk. Three factors were chosen (refrigerant temperature, stirring speed, and assay time) and their respective effects on the responses (concentrate yield, efficiency, concentration index, and average ice growth rate) were investigated. Aiming to enhance the separation efficiency of the system, a second stage was studied using the BFC assisted by vacuum. Besides, freezing point depressions of lactose-free milk and conventional milk were studied.

2. MATERIAL AND METHODS

2.1 MATERIAL

UHT lactose-free skim milk (CARREFOUR®, Madrid, Spain) was employed in the freezing concentration processes. It was purchased from a local supermarket in the area of Barcelona (Spain). Its initial composition was: 8.80 g 100 g^{-1} of total solids, 4.8 g 100 g^{-1} of carbohydrates (lactose <0.01%), 3.2 g 100 g^{-1} of proteins, and lipid <0.5 g 100 g^{-1}.

The same version of the product but with lactose (that is, UHT skim milk) was used for the preparation of concentrated milk, to investigate the behavior of both kinds of milk (with lactose and free-lactose) during freezing, and thus, verify the influence of lactose in this process. So, the UHT skim milk composition was 4.8 g 100 g^{-1} of carbohydrates, 3.3 g 100 g^{-1} of proteins, and < 0.5 g 100 g^{-1} of lipids.

2.2 PHYSICOCHEMICAL ANALYSIS

The total solids content of initial lactose-free milk, concentrated milk fractions, and ice fractions were determined according to Floren *et al*. (2016). Therefore, a standard curve of total solids content against °Brix readings was plotted employing distinct concentrations of lactose-free skim milk. For this purpose, milk samples were freeze-dried and the resulting powder was used to manufacture solutions with different concentrations (5%, 10%, 15%, 20%, 25%, 30%, 35%, and 40%). The °Brix readings of the solutions were performed using an Atago refractometer (DBX-55, Japan) with an accuracy of 0.1. Thus, through a linear regression ($y = 0.8715x - 0.3553$, $R^2 = 0.999$), the °Brix results of the tests were converted and demonstrated as total solids content (g 100 g^{-1}). Machado Canella *et al*. (2020) applied this same correlation to semi-skimmed goat milk ($y = 0.9285x + 0.2764$, $R^2 = 0.999$). In cases of readings of °Brix ≥ 4, the equation found by these authors shows similarity to our equation. That is, from this reading range, the estimated values for total solids will be smaller than their corresponding °Brix.

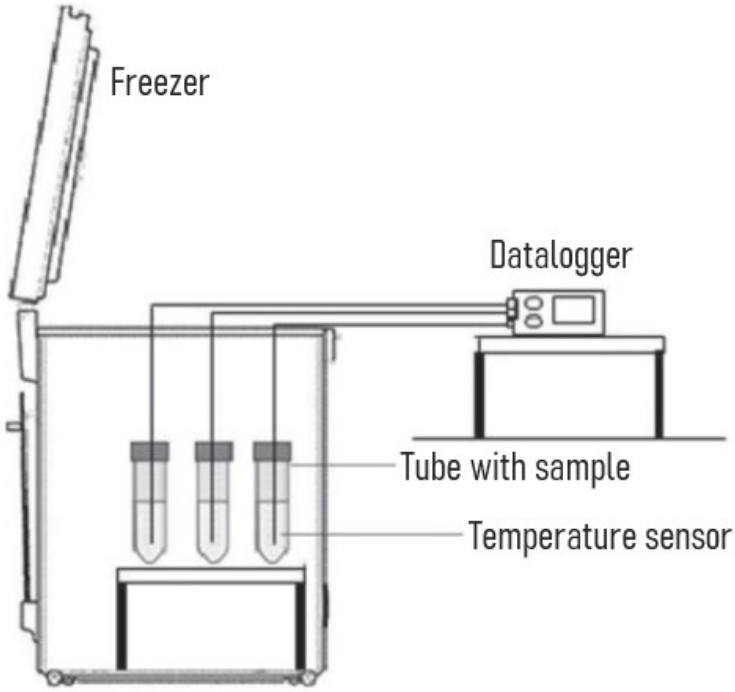
Protein contents (g 100 g^{-1}) were determined by the Kjeldahl method (N x 6.38) (AOAC, 2005), where N is the total nitrogen obtained by the method, and 6.38 is the conversion factor generally used for milk and dairy products. This multiplication gives us the amount of protein of the sample. Carbohydrates analyses (galactose, glucose, and lactose) were realized according to the methodology established by Schuster-Wolff-Bühring, Michael, and Hinrichs (2011), with modifications. An aliquot of 1 mL of sample was diluted with 8 mL of distilled water and vortexed. Then, 0.5 mL of Carrez Reagent 1 and 2 were added and the solution was mixed for 1 min. This mixture was allowed to settle for 15 min, and thereafter, filtered by a nylon syringe filter (0.45 μm of diameter pore) (Agilent, Santa Clara, California, United States). Twenty microliters of each sample were injected onto a carbohydrate column (ION 300) (Interaction Chromatography, San Jose, CA, USA) of an HPLC system (Hewlett Packard Series 1100, Agilent Technologies, Waldbronn, Germany). As a detector, it used a refraction index (Detector Beckman 156, San Ramon, California, United States). The mobile phase employed

was a sulfuric acid solution (0.013 M), with a flow rate of 0.4 mL min^{-1} at a temperature of 28 °C.

2.3 FREEZING POINT DEPRESSION

Aiming to observe the influence of lactose and/or simpler sugars (galactose and glucose) on the freezing point of milk, lactose-free skim milk and skim milk in different concentrations (5%, 10%, 15%, 20%, 25%, 30%, 35%, and 40%) were submitted to freezing. For this, the experimental apparatus used (Figure 10) consisted of three tubes with 40 mL of sample placed in a chest freezer (Fricon Model THC 520 N1, Portugal) at −20 °C. A type K thermocouple TESTO model 177-T4 (TESTO, Germany), previously calibrated with distilled water, was put in the middle of the closed tubes to register the temperature changes at intervals of 1 min.

Figure 10 − Apparatus to determine the freezing points of the skim milk and skim lactose-free milk under different concentrations.



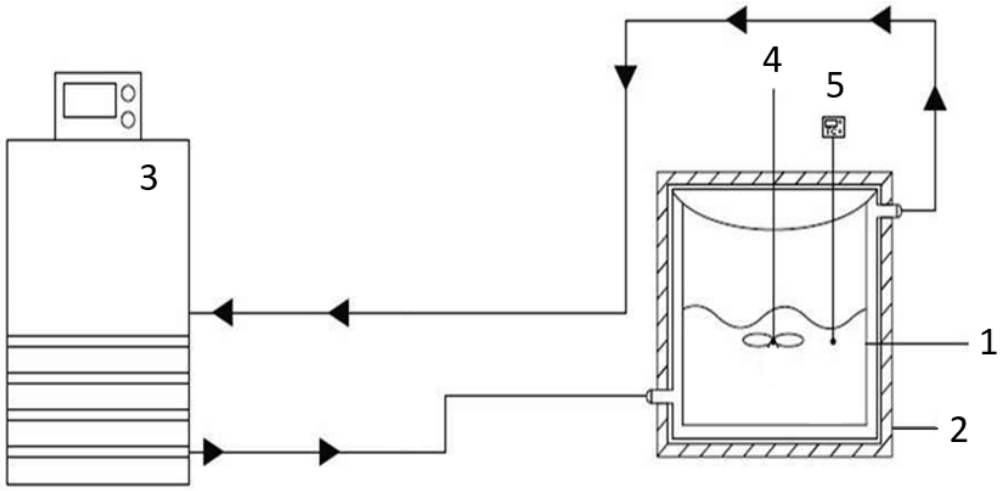
2.4 FREEZE CONCENTRATION SYSTEMS OF LACTOSE-FREE SKIM MILK

Two freeze concentration protocols (progressive freeze concentration and vacuum-assisted block freeze concentration) were used for the lactose-free milk concentration.

2.4.1 Experimental set-up of the progressive freeze concentration

The tests of progressive freeze concentration were performed in an equipment as described by Muñoz *et al*. (2018b) (Figure 11). In the receiving jacketed tank (1), 1.25 L of a previously refrigerated sample was placed. The tank, which has a total height of 230 mm and a diameter of 115 mm, was isolated with polyurethane foam (2) to limit heat exchange. The refrigerant fluid was composed of an ethylene glycol−water mixture (50% w/w) circulating in the thermostatic bath (3) (Polyscience SD15R-30, USA) which allows for maintaining the temperature between –30°C and 170°C ± 0.1°C and also have a temperature control system. The milk was agitated employing a mechanical stirrer (4) RGL-100 (Heidolph Instruments, Germany) equipped with a speed control system PCE-DT62 (PCE Deutschland GmbH, Germany) with 0.05% of accuracy and 0.1 rpm of resolution, and speed range: 250-5000rpm. During the tests, the sample temperature was registered using a digital Dattaloger Testo 925 (TESTO, Germany) (5) provided with a type K thermocouple with precision of 0.1 °C. The ice layer was formed in the walls of the vessel, and at the end of each test, the concentrated liquid was removed to separate it from the ice.

Figure 11 – Experimental set-up for progressive freeze concentration.



2.4.2 Experimental design for progressive freeze concentration

The response surface methodology (RSM) was employed to determine the most promising conditions for lactose-free milk progressive freeze concentration. Firstly, we took

into account a first-order model, which was a 2^3 factorial design augmented by six central points. The following independent factors were studied: stirring rate (ω) (500, 1000, and 1500 rpm), coolant temperature (*T*) (−5, −10, and −15 °C), and time of assay (*t*) (20, 40, and 60 min). Once the significant effect of the factors was confirmed, the design was extended to a central composite design (CCD) (second-order model). For this, we augmented the design with six axial points (face-centered). The resulting factorial CCD for the two-level and three-factor scheme with 20 treatments (three replicates) in total is described in Table 14.

After assessing the fit of the initial regression model, the number of variables was reduced following the stepwise method. This is a tool used to simplify the initial model and to find a reduced model that best explains the data. The reduced models were obtained with α to enter and α to remove equal 0.15. All of these analyzes were performed using Minitab 19 for Windows (Minitab Inc. Stage Collage, PA, USA). Thus, the regression coefficients were used to make response surfaces, and the results were reported as a mean ± standard deviation.

2.4.3 Response variables of the progressive freeze concentration

The response variables analyzed were the concentrate yield (*Y*), efficiency of the process (*eff*), concentration index (*CI*), and average ice growth rate ($\overline{v}_{ice}$).

The concentrate yield was calculated according to Moreno *et al*. (2014a), using Equation (1).

$$Y(\%) = \frac{C_{\mathrm{f}}\ m_{\mathrm{f}}}{C_0\ m_0}\ x\ 100 \tag{1}$$

where C_{f} is the total solids content of the concentrate fraction (g 100 g^{-1}), C_0 is the initial total solids content of the lactose-free skim milk (g 100 g^{-1}), m_{f} is the concentrate fraction mass (g), and m_0 is the initial milk mass (g).

The efficiency of the progressive freeze concentration relates to the increase in the solids concentration of the concentrate fraction relative to the solids content retained in the ice fraction. Thus, this parameter was determined using Equation (2):

$$eff(\%) = \frac{C_{\mathrm{f}} - C_{\mathrm{i}}}{C_{\mathrm{f}}}\ x\ 100 \tag{2}$$

where C_f is the total solids content of the concentrate fraction (g 100 g^{-1}), and C_i is the total solids content (g 100 g^{-1}) of the ice fraction.

The concentration index was defined as the relation between the content of total solids in the concentrate fraction and the total solids content of the initial milk. As mentioned by Nakagawa, Maebashi, and Maeda (2009), this parameter is also known as relative concentration (Equation 3).

$$CI = \frac{concentrate\ fraction\ total\ solids\ (\text{g } 100\ \text{g}^{-1})}{initial\ total\ solids\ (\text{g } 100\ \text{g}^{-1})} \quad (3)$$

The average ice growth rate was measured at the end of the experiment and takes into account the concentration, density, and mass of the ice, as well as the heat transfer area and time of the experiment, as shown in Equation 4 (Osorio *et al*., 2018).

$$\overline{v}_{ice}(\mu\text{m s}^{-1}) = \frac{r - \sqrt{r^2 - \frac{m_i\,(1 - C_i)}{\rho_i\,h\,\pi}}}{t}\ 10^6 \quad (4)$$

where r is the vessel radius (m), m_i is the mass of the ice sheet (kg), h is the ice layer height (m), ρ_i is the ice density (kg/m^3), C_i is the total solids content (g 100 g^{-1}) of the ice fraction, and t is the time of assay (s).

Finally, the ice fraction of each test was reported to better understand the results. For this proposal, Equation 5 was used:

$$\text{Ice fraction (\%)} = \frac{ice\ mass\ (g)\ x\ 100}{initial\ mass\ (\text{g})} \quad (5)$$

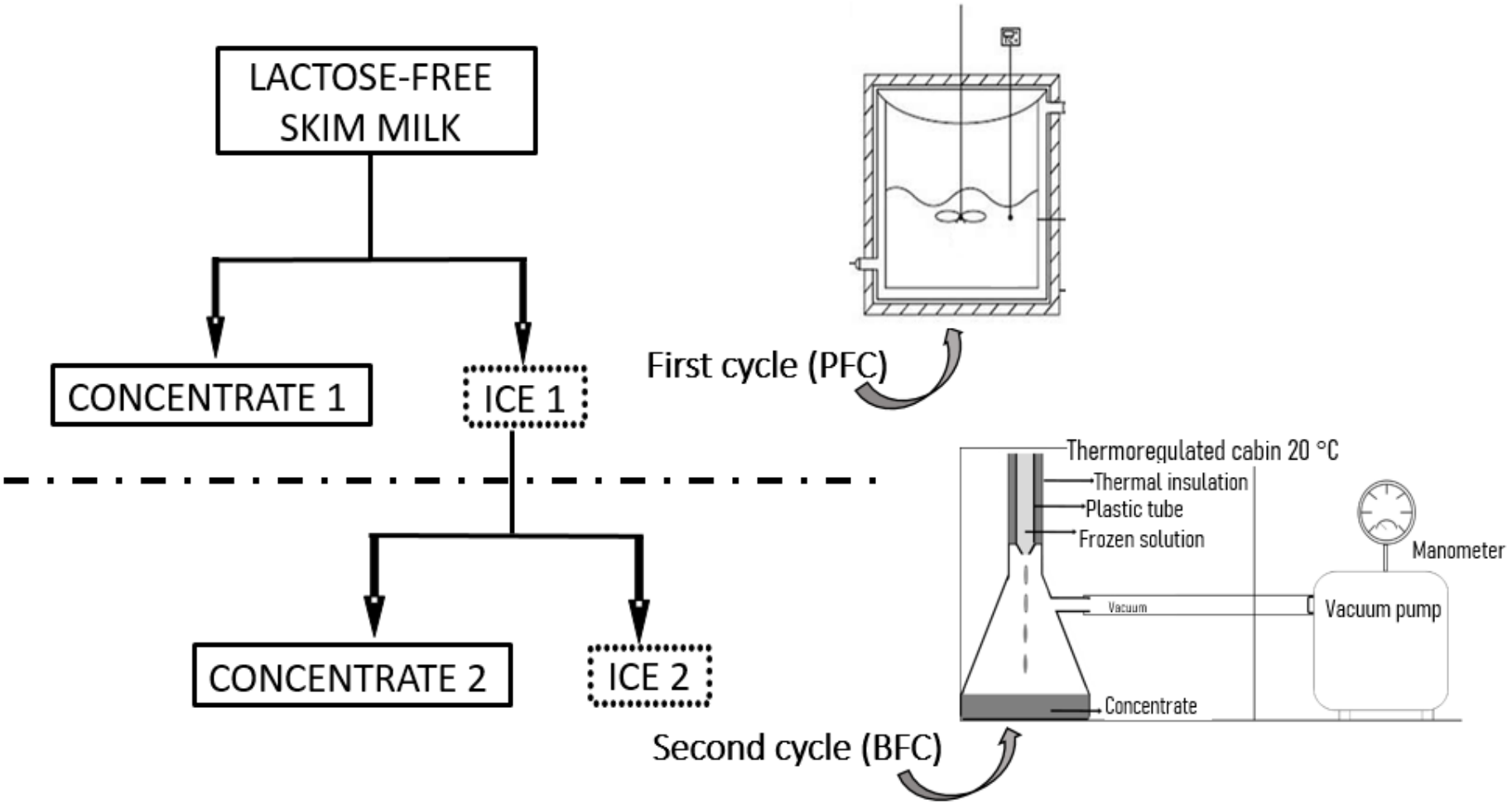
2.4.4 Vacuum-assisted block freeze concentration

Under the best condition for *Y* and *eff* (time of 58 min, stirring rate of 1035 rpm, and coolant temperature of –5 ºC), ice samples were submitted to the vacuum-assisted block freeze concentration (Figure 12), following the methodology described by Machado Canella *et al*. (2020), with modifications. For this, the ice obtained from progressive freeze concentration was completely melted at room temperature. Then, the liquid was placed in plastic tubes (45 mL)

enveloped with polystyrene foam and frozen in a chest freezer at –20ºC for 18 hours. After the freezing process, the vacuum was applied on the condition of 10 kPa (absolute pressure) for 65 min. The choice of these parameters was based on correlated works. For example, Hernández *et al*. (2021) verified the best separation conditions at 10 kPa and longer test times. Machado Canella *et al*. (2020), who studied the freeze concentration of goat milk, found an optimal condition using the vacuum equal to 10 kPa and a time of 60 min. In our experiment, the suction was produced by plugging a vacuum pump into the bottom of the frozen sample. This procedure was replicated 3 times. In summary, we considered the PFC as the first cycle of lactose-free milk freeze concentration (the concentrated and ice fractions were denoted as Concentrate 1 and Ice 1, respectively). The second cycle consisted of BFC assisted by vacuum, where its fractions were denoted as Concentrate 2 and Ice 2. Equation 6 was used to calculate the *CI* of the second cycle:

$$CI = \frac{Concentrate\ 2\ total\ solids\ (\mathrm{g\ 100\ g^{-1}})}{Ice\ 1\ total\ solids\ (\mathrm{g\ 100\ g^{-1}})} \quad (6)$$

Figure 12 – Freeze concentration schematic diagram of lactose-free milk in two steps.

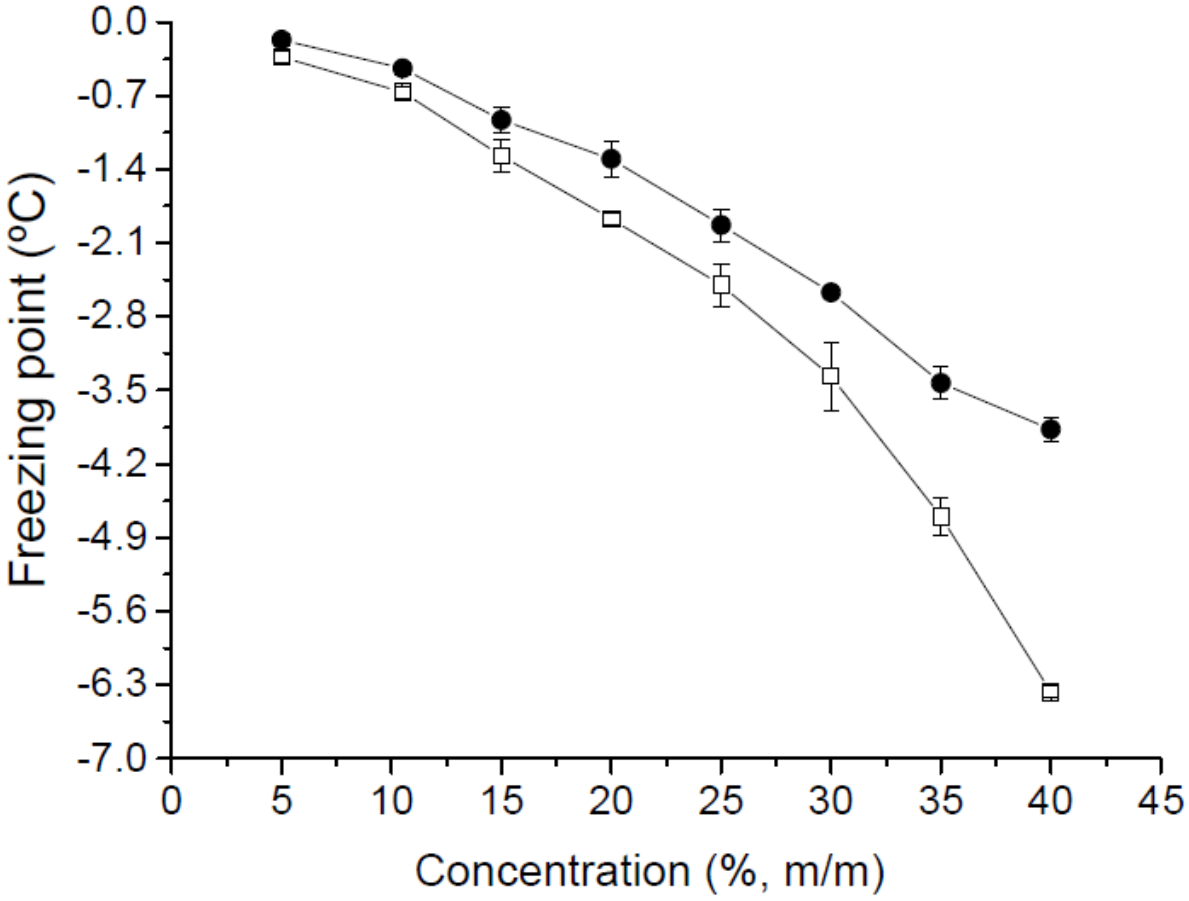


3. RESULTS AND DISCUSSION

3.1 FREEZING POINT DEPRESSION

Figure 13 shows the freezing points of both kinds of milk (with lactose and free-lactose) under different concentrations. Hernández *et al*. (2009) commented that the freezing point of a liquid food depends on the types of solutes present in the solution and their respective concentrations. Thus, more concentrated solutions commonly have a lower freezing point, exactly as observed in our samples. We have performed the Student t test of two means and statistically significant differences ($P = 0.021$) appear between the freezing points of milk with and without lactose. For skim milk, the freezing point dropped from – 0.17 °C ± 0.06 (concentration of 5%) to – 3.87 °C ± 0.12 (concentration of 40%). Lide (1998) reported a freezing point depression of 0.288 °C for a lactose concentration of 5%. Small differences were expected, since skim milk not only contains lactose, it also contains caseins and whey proteins, which alter the freezing point properties (MA; BARBANO, 2003). In its natural concentration (~ 10%), skim milk presented a freezing point of – 0.43 °C ± 0.06, which is similar to the result reported by Muñoz *et al*. (2018b) also for skim milk. On the other hand, the freezing point of the lactose-free skim milk dropped from – 0.33 °C ± 0.06 (concentration of 5%) to – 6.37 °C ± 0.06 (concentration of 40%). As informed by the manufacturer, the lactose-free skim milk used in this study was lactose-free due to previous enzymatic hydrolysis with β-galactosidase. Thereby, the resulting milk contains large amounts of the monosaccharides glucose and galactose (approximately 2.4 g 100 g^{-1} of each), which contributes to the increase of its overall molar concentration of sugars (Churakova et al., 2019). As result, a linear decrease in the freezing point was observed. The findings of De Oliveira Neves and Leal de Oliveira (2021) showing similarities, that is, lactose-free milk presented a lower freezing point than its non-hydrolyzed version. Muñoz *et al*. (2018b) highlighted that through the determination of the freezing point, best working conditions for the freeze concentration process can be established.

Figure 13 – Freezing points of the skim milk (●) and skim lactose-free milk (□) under different concentrations.



3.2 PROGRESSIVE FREEZE CONCENTRATION

The experimental data corresponding to the average concentrate yield (*Y*), *eff*, *CI* and $\overline{v}_{ice}$ are shown in Table 14. The *P* values of the reduced model and the initial model are presented in Table 15, which shows that for all responses, the individual effects of the factors were significant ($P < 0.05$). These models were obtained after applying RSM.

A regression equation (Equation 7) for *Y* was obtained from the reduced model with a coefficient of determination (R^2) equal to 0.982. Analyzing this model, we observed that only one quadratic term was significant ($P \leq 0.05$) (ω^2). Regarding the interaction between factors, they all had a significant effect ($P \leq 0.05$) on the concentrate yield.

$$Y = 76.68 + 0.015T + 0.03339\omega + 0.3160t - 0.000012\omega^2 + 0.00107T\omega + 0.04739Tt - 0.000275\omega t \quad (7)$$

where T is the coolant temperature (°C), ω is the stirring rate (rpm), and t is the time (min) of assay.

In Figure 14(a), the contour plot illustrates the variation of Y with the interaction factors (time and temperature). There was an increase for the Y value when higher coolant temperatures were used, together with a shorter test time. Muñoz *et al*. (2018b), who studied the progressive freeze concentration of skim milk, also reported better concentrate yield using a coolant temperature of – 5 °C. As can be seen in Table 14, in general, it happens that when CI is maximum, Y is minimum. This is related to the amount of ice produced. For example, at –15 °C, 1500 rpm and 60 min (Y = 25.26%; CI = 1.7), an ice fraction of 83.8% is obtained. Meanwhile, at – 5 °C, 500 rpm, and 20 min (Y = 87.8%; CI = 1.07) only 17.3% of ice fraction is produced. For this reason, the liquid has more solids from the initial milk but it is less concentrated (lower CI). Thus, an optimized value of Y might not correspond to an efficient process of freeze concentration. In contrast, at –15 ºC, 1500 rpm and 60 min, Y is minimum and IC is maximum. That is, the final concentration is the highest of the Table 14, but the low value of Concentrate Yield would indicate that the solutes present in the solution decreased and were occluded in the ice crystal (OSORIO *et al*., 2018). This phenomenon is due to a higher ice growth rate ($\overline{v}_{ice}$) in this condition, because of the lower temperature on the surface of the vessel. Muñoz *et al*. (2018a) and Muñoz *et al*. (2018b) investigated the PFC for solutions of whey and skim milk, respectively. These solutions had lower initial concentration compared to our lactose-free milk samples (7.66 °Brix and 5.00 g 100 g^{-1} of total solids, respectively; against ≃ 8.88 g 100 g^{-1} of total solids or 10.6 °Brix for lactose-free milk samples). Moreno *et al*. (2014b) and Robles *et al*. (2016) explained that a higher initial concentration implies an increase of viscosity, which in turn hinders mass transfer. This condition induces the formation of dendritic ice, generating occlusions. Osorio *et al*. (2018) found the higher Y values for ethanol-water solutions at the lowest concentration (3% w/w) studied. Ethanol-water solutions were freeze concentrated in an apparatus similar to that used in our work.

Table 14 – Central Composite Design (CCD) for three variables levels and their responses of Concentrate yield, Efficiency, Concentration index, Average ice growth rate, and Fraction of ice.

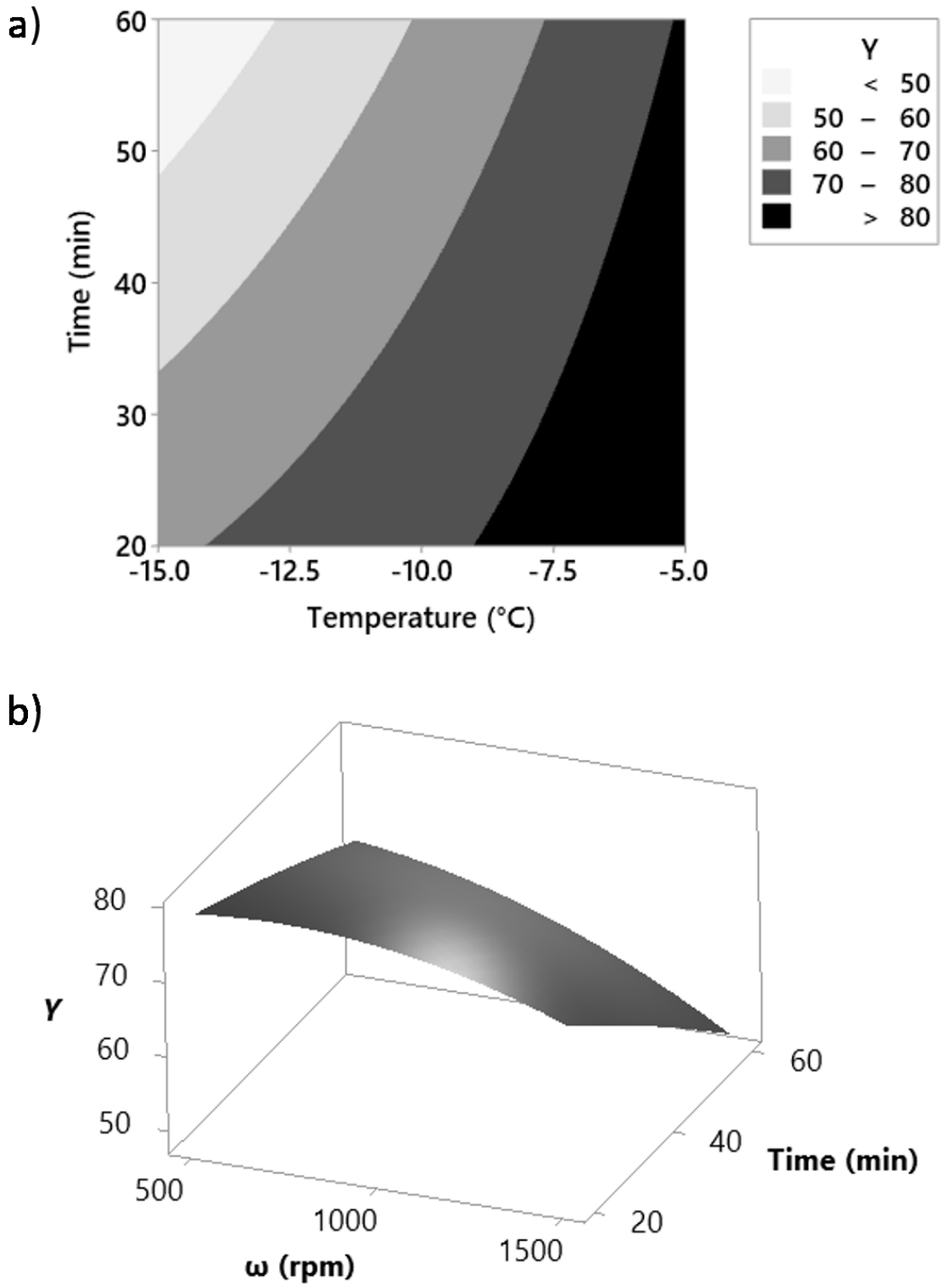
	Variables levels*			Response				
Type	Temperature (°C)	ω (rpm)	Time (min)	*Y* (%)	*eff* (%)	*CI*	$\overline{v}_{ice}$ (μm/s)	Ice fraction (%)
Factorial	–5 (1)	500 (–1)	20 (–1)	87.81 ± 0.72	36.30 ± 1.72	1.07 ± 0.01	3.05 ± 0.27	17.34 ± 1.23
	–5 (1)	500 (–1)	60 (1)	82.91 ± 2.13	51.93 ± 0.23	1.20 ± 0.01	1.85 ± 0.13	29.82 ± 2.01
	–5 (1)	1500 (1)	20 (–1)	84.59 ± 2.10	47.59 ± 1.09	1.13 ± 0.02	3.74 ± 0.35	24.03 ± 2.08
	–5 (1)	1500 (1)	60 (1)	72.71 ± 4.26	60.90 ± 1.52	1.34 ± 0.04	2.41 ± 0.15	45.07 ± 1.84
	–15 (–1)	500 (–1)	20 (–1)	70.02 ± 1.57	32.39 ± 1.83	1.12 ± 0.00	6.94 ± 0.32	37.24 ± 1.58
	–15 (–1)	500 (–1)	60 (1)	50.18 ± 0.88	45.87 ± 1.07	1.42 ± 0.02	4.23 ± 0.08	63.84 ± 0.92
	–15 (–1)	1500 (1)	20 (–1)	60.11 ± 2.62	41.74 ± 1.44	1.26 ± 0.04	8.31 ± 0.09	51.49 ± 0.81
	–15 (–1)	1500 (1)	60 (1)	25.26 ± 0.61	51.66 ± 0.80	1.70 ± 0.08	4.76 ± 0.03	83.82 ± 0.48
Center	–10 (0)	1000 (0)	40 (0)	69.66 ± 0.78	52.34 ± 1.48	1.34 ± 0.02	4.29 ± 0.19	47.19 ± 0.98
	–10 (0)	1000 (0)	40 (0)	67.93 ± 1.74	53.85 ± 1.07	1.37 ± 0.01	4.59 ± 0.03	49.35 ± 1.04
	–10 (0)	1000 (0)	40 (0)	68.79 ± 1.17	55.01 ± 1.65	1.38 ± 0.00	4.53 ± 0.11	49.49 ± 0.55
	–10 (0)	1000 (0)	40 (0)	70.54 ± 2.24	55.36 ± 1.83	1.36 ± 0.02	4.29 ± 0.10	47.46 ± 2.65
	–10 (0)	1000 (0)	40 (0)	70.64 ± 1.21	54.53 ± 0.93	1.36 ± 0.01	4.29 ± 0.09	47.05 ± 0.92
	–10 (0)	1000 (0)	40 (0)	69.26 ± 0.98	53.43 ± 0.90	1.36 ± 0.01	4.41 ± 0.06	48.16 ± 0.79
Axial	–5 (1)	1000 (0)	40 (0)	84.25 ± 1.74	55.89 ± 1.00	1.20 ± 0.01	2.52 ± 0.15	28.63 ± 1.57
	–10 (0)	500 (–1)	40 (0)	74.32 ± 1.06	43.66 ± 0.24	1.22 ± 0.01	3.66 ± 0.15	38.82 ± 1.51
	–10 (0)	1000 (0)	20 (–1)	77.39 ± 0.21	43.21 ± 1.18	1.17 ± 0.01	5.88 ± 0.04	33.50 ± 0.34
	–10 (0)	1000 (0)	60 (1)	62.32 ± 0.58	59.18 ± 0.58	1.56 ± 0.01	3.71 ± 0.06	59.32 ± 0.76
	–10 (0)	1500 (1)	40 (0)	60.35 ± 0.41	52.22 ± 1.03	1.41 ± 0.04	4.60 ± 0.01	56.28 ± 1.36
	–15 (–1)	1000 (0)	40 (0)	57.65 ± 1.29	52.26 ± 1.70	1.46 ± 0.02	5.62 ± 0.17	60.12 ± 1.51

Table 15 – *P* Values of ANOVA test from the regression of the initial and reduced models.

Term		*P* Values			
		Y	*eff*	*CI*	$\overline{\nu}_{ice}$
Complete model	Constant	0.000*	0.000*	0.000*	0.000*
	T (°C)	0.000*	0.000*	0.000*	0.000*
	ω (rpm)	0.000*	0.000*	0.000*	0.000*
	t (min)	0.000*	0.000*	0.000*	0.000*
	T^2	0.418	0.477	0.007*	0.000*
	ω^2	0.000*	0.000*	0.000*	0.003*
	t^2	0.453	0.000*	0.751	0.000*
	*T*ω	0.000*	0.033*	0.000*	0.032*
	Tt	0.000*	0.022*	0.000*	0.000*
	ω*t*	0.000*	0.015*	0.000*	0.002*
Reduced model	Constant	0.000*	0.000*	0.000*	0.000*
	T (°C)	0.000*	0.000*	0.000*	0.000*
	ω (rpm)	0.000*	0.000*	0.000*	0.000*
	t (min)	0.000*	0.000*	0.000*	0.000*
	T^2	–	–	0.005*	0.000*
	ω^2	0.000*	0.000*	0.000*	0.003*
	t^2	–	0.000*	–	0.000*
	*T*ω	0.000*	0.032*	0.000*	0.032*
	Tt	0.000*	0.021*	0.000*	0.000*
	ω*t*	0.000*	0.014*	0.000*	0.002*

–: Term no applied in the model.
*Significant term ($P \leq 0.05$)

Figure 14 – (a) Contour plot of the response Yield (*Y*) at 1000 rpm, and (b) surface plot of the interaction effect of time and agitation speed at –10 °C.



When evaluating the interaction time-agitation speed (Figure 14-b), the best concentrate yields were noted for the lower agitations with shorter test times. Under these conditions, the highest solute yield values are obtained, due to the lower production of ice. Moreover, it is observed that the effect of agitation speed is greater the longer the test time. The findings of Muñoz *et al*. (2018b) show a clear dependence on the agitation, with the maximum solid recovery, obtained also at 500 rpm. On the other hand, the agitation (500–2000 rpm) did not have a significant effect on the *Y* values for the progressive freeze concentration of ethanol-water solutions (OSORIO *et al*., 2018).

The final regression equation (R^2 = 0.967) correlating *eff* with the independent variables is as follows:

$$eff = -3.14 + 0.041T + 0.0594\omega + 0.962t - 0.000023\omega^2 - 0.00597t^2 + 0.000255T\omega + 0.00692Tt - 0.000074\omega t \quad (8)$$

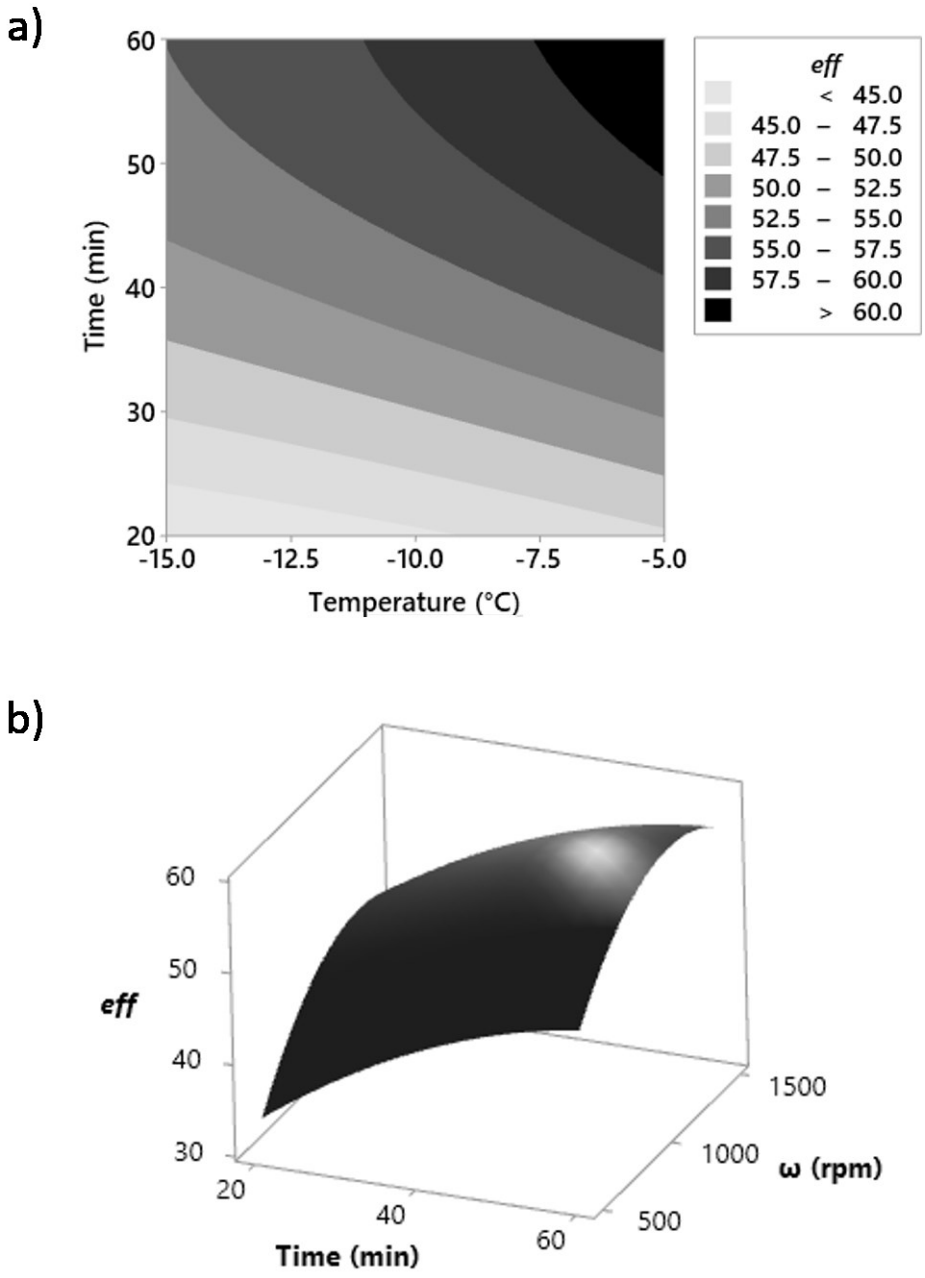
This reduced model shows us that all interactions between factors affected ($P \leq 0.05$) the efficiency. The quadratic terms of stirring rate and time also were significant ($P \leq 0.05$), whereas the quadratic term of temperature was not significant ($P > 0.05$). Figure 15(a) shows that the best *eff* values were found when used higher coolant temperatures and longer experiment times. Moreover, high agitation speeds also favored the achievement of highest *eff* values (Figure 15-b). We believe that the dependence of ω is because the higher the rotation speed, the greater the tendency of the solids to remain distant from the ice-solution interface, and thus not to be retained during the crystallization process. Furthermore, Liu, Miyawaki, and Hayakawa (1999) affirmed that the fluid stirring enhances the mass transfer rate of the solutes from the ice surface towards the liquid fraction.

It is important to highlight that in the calculation of efficiency, the total solids present in the ice are taken into account. Obviously, it is ideal that the ice obtained to have the lowest concentration of total solids. Our results denote that the amount of solids in the ice is defined by the agitation rate and by the advance of the ice front. In this context, Shirai *et al*. (1998) affirmed that by diminishing the advance of the ice and increasing the agitation, the ice concentration decreases.

The Concentration Index is a measure of the final concentration of solids in the liquid. Analyzing the reduced model (Table 15 and Equation 9) of this response variable, we see that all the effects were significant ($P \leq 0.05$) except the quadratic term of time (t^2) ($P > 0.05$). The model presented a coefficient of determination (R^2) equal to 0.958.

$$CI = 0.7892 - 0.01607T + 0.000339\omega - 0.00038t - 0.001275T^2 - 0.0000001\omega^2 - 0.000011T\omega - 0.000486Tt + 0.000003\omega t \quad (9)$$

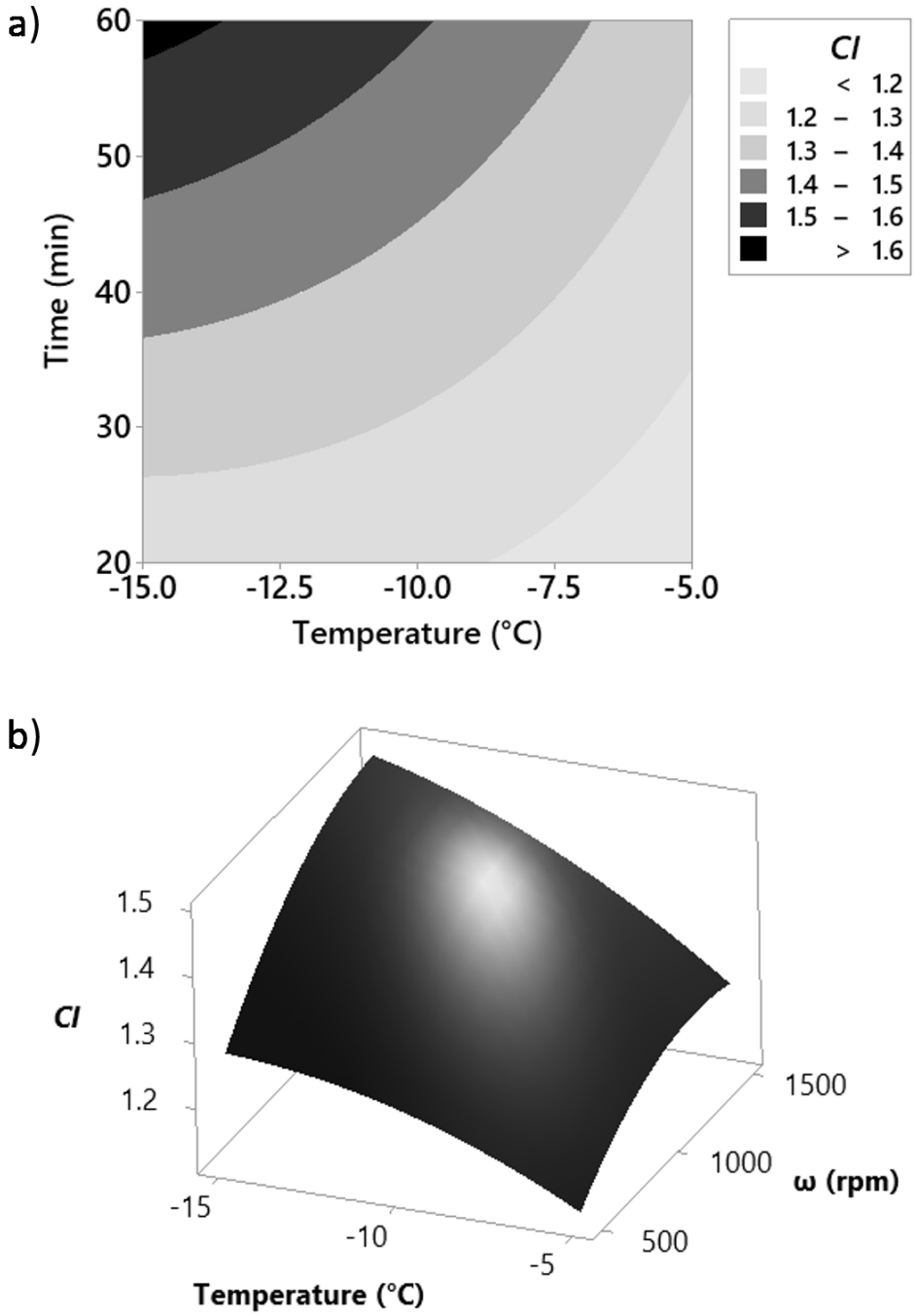
Figure 15 – (a) Contour plot of the response Efficiency (*eff*) at 1000 rpm, and (b) surface plot of the interaction effect of time and agitation speed at –10 °C.



The *CI* was maximized at low temperatures, longer experiment times (Figure 16-a), and at high agitation speeds (Figure 16-b). The highest *CI* value of 1.7 corresponds to the condition –15ºC / 1500rpm / 60 min (Table 14). This is due to the large amount of ice produced

under these conditions (83.8% ice fraction). Likewise, the $\overline{v}_{ice}$ of 8.31 μm s^{-1} is the greatest of all the conditions tested. These results are in conformity with those reported in previous works using the same freeze concentration system with sucrose solutions (OJEDA *et al*., 2017; MOUSSAOUI *et al*. 2018) and skim milk (MUÑOZ *et al*., 2018b). In both cases, the maximum concentration was obtained with the minimum temperature and maximum agitation tested, although in these works the variation of the time of experiment was not evaluated. Particularly, in the study of Muñoz *et al*. (2018b), the maximum *CI* reached was 1.72, using a stirring of 1000 rpm, –15 ºC of temperature, and time set at 60 min. On the other hand, the least *CI* values of our work were observed for the highest *T* and milder ω. Ojeda *et al*. (2017) related that the decrease in ω can reduce the mass transfer; moreover, the higher the coolant temperature, the lower the heat transfer in the process. The authors emphasized that this combined effect decrease the ice growth rate (as can also be seen in our results in Table 14).

Figure 16 – (a) Contour plot of the response Concentration index (*CI*) at 1000 rpm, and (b) surface plot of the interaction effect of temperature and agitation speed in assays of 40 min.



As can be seen in Table 15 and Equation 10, all the effects (linear, quadratic, and of interaction) for the average ice growth rate were significant ($P \leq 0.05$). The reduced model presented R^2 equal to 0.987.

$$\overline{v}_{ice} = 0.577 - 0.6802T + 0.002533\omega - 0.091t - 0.00999T^2 - 0.000001\omega^2 + 0.001191t^2 - 0.000032T\omega + 0.004678Tt - 0.000012\omega t \quad (10)$$

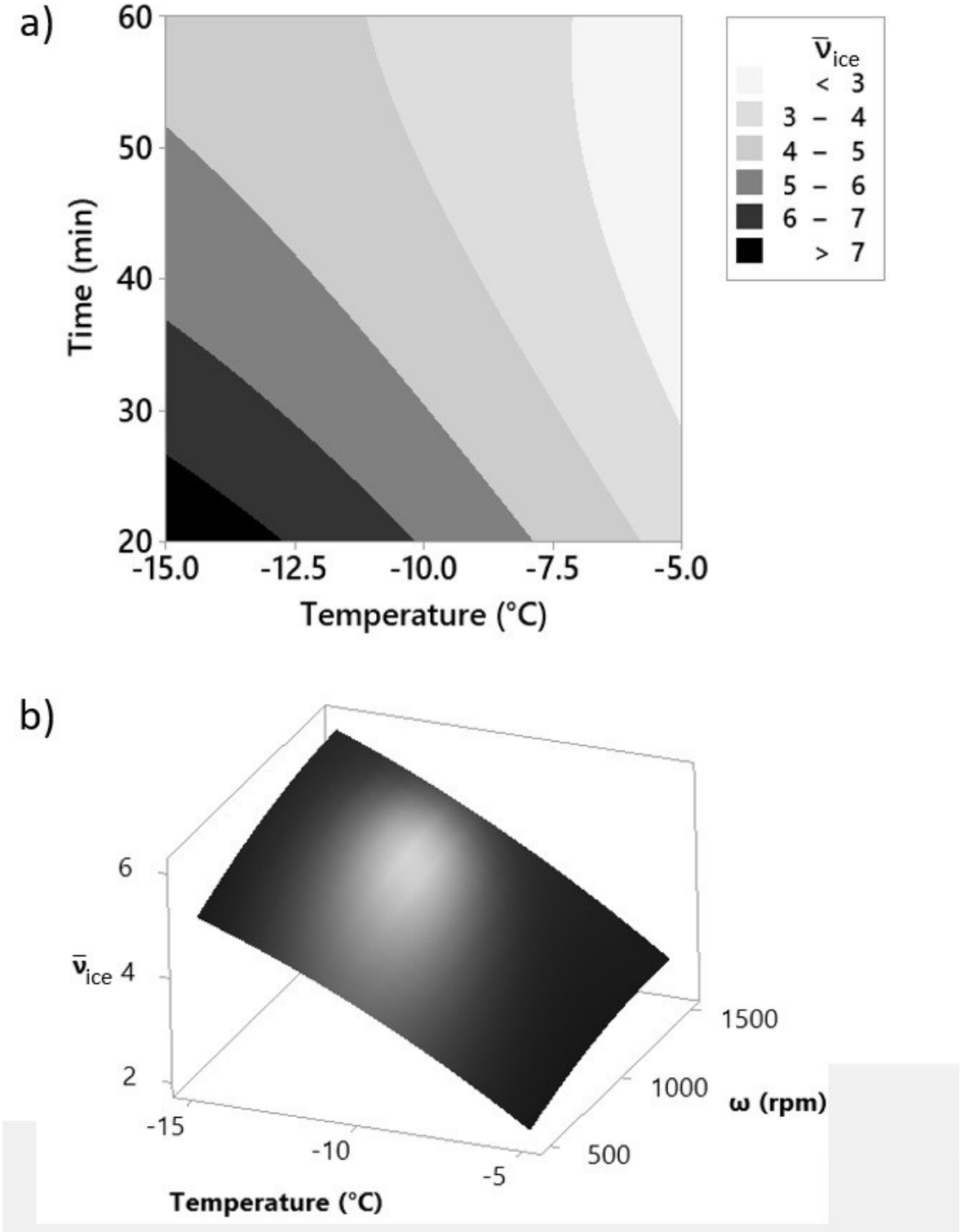
For a same temperature and stirring, the average ice growth rate decreased with increasing time. This occurs because of the resistance to heat transfer present in the system. As time increases, there is a continuous increment of ice on the reactor wall. This sheet of ice difficulties the transfer of heat between the refrigerant and the solution to be concentrated, decreasing the average ice growth rate.

The response contour of $\overline{v}_{ice}$ as a function of the temperature and time (Figure 17-a) also reveals a marked dependence on the temperature of the refrigerant. That is, the lower the coolant temperature, the higher the $\overline{v}_{ice}$. An example can be observed when comparing the responses of condition at –5 ºC, 1500 rpm, and 20 min with the condition at –15 ºC, 1500 rpm, and 20 min (Table 14). The $\overline{v}_{ice}$ was more than double when the temperature level changed from –5 to –15 ºC, reaching the highest value of the table (8.31 μm s^{-1}). However, it was not observed such a marked increase in the concentration index. In contrast, *eff* and *Y* values decreased, indicating greater migration of the solutes onto the crystalline phase. The influence of temperature on $\overline{v}_{ice}$ has also been reported in previous works of freeze concentration systems (MORENO *et al*., 2014b; OSORIO *et al*., 2018; OJEDA *et al*., 2017; VUIST; BOOM; SCHUTYSER, 2020). Some authors established that an average ice growth rate greater than 8 μm s^{-1} is very fast to achieve an effective separation of the concentrated phase (MORENO *et al*., 2014a; PETZOLD *et al*., 2016). Robles *et al*. (2016) and Moreno *et al*. (2014b) discussed that at low ice growth rates, the expulsion of solutes from the formed network is favored due to the more orderly growth of the crystal. Besides everything, the average ice growth rate increased slightly with increasing fluid agitation (Figure 17-b).

When applying the Response Optimizer function (software Minitab) for the progressive freeze concentration data, the maximum values of *Y* and *eff* would be 80.81% and 62.22%, respectively. According to the model, these values can be achieved at – 5 ºC, 58 min and 1035 rpm. Therefore, we performed three experiments under these conditions and found values of *Y*, *eff*, *CI*, and $\overline{v}_{ice}$ equals to 81.92 ± 1.57, 60.64 ± 0.30, 1.34 ± 0.03, and 2.94 ± 0.04, respectively. For these optimal conditions, the experimental result of the ice fraction obtained corresponds to 37%. This result is consistent with those reported by Muñoz *et al*. (2018b) with skimmed milk. In this work, the optimal condition for *Y* is obtained at –5 °C / 500rpm / 60min with 27% of ice formed. The difference with the results obtained in the optimal conditions of our work may be due to the greater agitation (1035 instead of 500 rpm), which improves the flow of water from the concentrated fraction to the ice fraction (mass transfer), and hence also

the heat transfer. These data are confirmed by comparing the average ice growth rate under optimal conditions of 1.55 μm s^{-1} in Muñoz *et al*. (2018b) with the 2.94 μm $s^{-1}$of our work.

Figure 17 – (a) Contour plot of the response Average ice growth rate (ν) at 1000 rpm, and (b) surface plot of the interaction effect of temperature and agitation speed in assays of 40 min.



The contents of glucose, galactose, lactose and protein of two cycles were measured and are presented in Table 16. In the PFC system, the concentration indices (*CI*) of total solids, carbohydrates (glucose, galactose and lactose) and proteins were calculated using eq. 3 applied between Concentrate 1 and the original composition of the milk (Table 16). Values of 1.33, 1.40 and 1.19 were obtained, respectively. These results suggest that in the PFC system the proteins have a greater tendency to remain in the ice, while the carbohydrates pass into the concentrated liquid phase. These results are in agreement with the work of Kawasaki, Matsuda and Kadota (2006). They studied progressive freeze concentration of multi-component solutions. They found that the small molecular weight solutes separated and concentrated more efficiently than the higher molecular weight solutes, and this corresponded well with the magnitude of the diffusion coefficient of each solute. Similar results have been reported by Nakagawa *et al*. (2010). They observed that phenol (M.W = 94 g/mol) was more concentrated in the liquid zone than dye (M.W = 993 g/mol). The difference of these freeze-concentration behaviors would be due to the difference of the mobility when the substances meet the freezing front. The mobility of the solutes is affected by the molecular size and the concentration.

3.3 VACUUM-ASSISTED BLOCK FREEZE CONCENTRATION

Given the considerable amount of total solids retained in the Ice 1 (Table 16), this fraction was thawed, frozen again, and subjected to the vacuum-assisted block freeze concentration (2nd cycle). The contents of glucose, galactose, lactose and protein of two cycles were measured and are presented in Table 16. In the BFC system, the concentration indices (*CI*) of total solids, carbohydrates (glucose, galactose and lactose) and proteins were calculated using eq. 6 applied between Concentrate 2 and the Ice 1 (Table 16). Unlike the PFC system, in the BFC tests the results obtained suggest that the protein is concentrated in the liquid phase (*CI* = 6.4), while the carbohydrates (*CI* = 4.3) follow the same trend as that of the total solids (*CI* = 4.0). These results are in agreement with those reported by Aider, de Halleux, and Akbache (2007) and Aider and Ounis (2012) for the block cryoconcentration of whey and skimmed milk, respectively. The thaw concentration mechanism has been extensively studied by Nakagawa *et al*. (2009). It was found that the solution obtained during thawing showed a higher concentration than the original solution. It also suggests that the concentration of the thawed solution could be greatly influenced by the morphology of the ice crystals. According to Yee *et al*. (2004), the growth of dendritic ice crystals is expected during the freezing of

carbohydrate solutions (glucose, fructose, sucrose and lactose), while in milk protein solutions the crystals grow smoothly (planar front). This softer morphology could favor the drainage of milk proteins towards the liquid phase. This behavior could explain the higher *CI* of proteins in relation to carbohydrates. Nevertheless, the concentration of total solids (*CI* = 4.00) achieved in the BFC test is considered high; this result and the low solids content verified in Ice 2 indicate that the BFC system is efficient for concentrating lactose-free milk components. Machado Canella *et al*. (2020) explained that this behavior is expected since the external driving force (vacuum) improves the natural division of gravitational thawing.

Table 16 – Carbohydrate and protein content (mean ± standard deviation) of lactose-free milk, and concentrates and ice fractions from first and second freeze concentration cycles.

	Lactose-free milk	Concentrate 1	Ice 1	Concentrate 2	Ice 2
Total solids (g 100 g^{-1})	8.8	11.68 ± 0.09	4.61 ± 0.15	18.47 ± 0.01	1.21 ± 0.01
Protein (g 100 g^{-1})	3.2	3.809 ± 0.319	1.050 ± 0.074	6.700 ± 0.104	0.521 ± 0.044
Glucose (g 100 g^{-1})	~ 2.4*	3.390 ± 0.008	1.215 ± 0.004	5.130 ± 0.019	0.339 ± 0.001
Galactose (g 100 g^{-1})	~ 2.4*	3.224 ± 0.075	1.160 ± 0.005	4.824 ± 0.028	0.359 ± 0.001
Lactose (g 100 g^{-1})	< 0.01	0.118 ± 0.003	0.024 ± 0.010	0.292 ± 0.011	< 0.01

Concentrate 1 and Ice 1 were fractions obtained by progressive freeze concentration at –5 ºC, 58 min and 1035 rpm. Concentrate 2 and Ice 2 are fractions obtained by vacuum-assisted block freeze concentration from Ice 1. *The manufacturer does not provide data for monosaccharides, but only for total carbohydrates (4.8 g 100 g^{-1}). Therefore, glucose and galactose contents were expressed as approximate values, as discussed in section *3.1 Freezing point depression* of this work.

The *CI* obtained with vacuum BFC was much higher than that of PFC (4 vs 1.33 average). This may suggest that it is better to apply vacuum BFCs to the starter milk, and not just the ice fractions. In our opinion, for practical applications, the parameter to be optimized is the solute yield (*Y*) instead of the concentration index (*CI*). As stated above, a high *CI* value implies a low *Y* value. This means that highly concentrated flows are produced, but in small quantities. Many valuable solutes remain in the ice, which must be properly managed for recovery. In this way, we can refer to the work of Petzold *et al*. (2016) on the application of vacuum-assisted BFC applied to wine. The authors consider for practical applications that the suitable vacuum time is 21 min, at a vacuum pressure of 40 kPa. Under these conditions, a moderate *CI* of 1.7 and a high *Y* value (82%) are obtained. In any case, the simultaneous optimization of these two parameters (*Y* and *CI*), using more advanced statistical techniques (desirability function), could be considered as lines of future work. These analysis techniques are outside the scope of this document.

On the other hand, for practical applications, time is an essential factor to consider. Thus, in PFC the process time in the optimal condition is 58 min. In the case of BFC, the time of vacuum applied (65 min) must be added that of freezing. For samples used in BFC, the freezing time, estimated using the Pham model (2008), is 300 min. Resulting in a total process time in BFC of 365 min. In this time, theoretically, up to 6 cycles of the PFC system can be carried out. In our work, the combination of the two techniques (PFC + Vacuum-assisted BFC) makes it possible to recover 97% of the initial solids, while improving the *CI* to 1.42. This idea of combining techniques has been suggested in previous works (HERNÁNDEZ *et al*. 2021), where can quickly remove ice using PFC, and then purify it efficiently using BFC. As confirmed by Canella *et al*. (2019), who freeze concentrated skim goat milk by BFC, the increasing of freeze concentration cycles may enhance the content of important minerals, such as calcium and magnesium. Moreover, it was reported that the use of unitary operations associated with low temperatures and vacuum resulted in a substantial increase in the taste and flavor of food products (SUN; ZHENG, 2006). So, it is expected that lactose-free milk submitted to these freeze concentration processes could have different sensorial properties.

4. CONCLUSIONS

The enzymatic hydrolysis of milk and the concentration of total solids altered its depression freeze point. While investigating the effect of process parameters on progressive

freeze concentration of lactose-free milk, we observed that all individual factors presented a significant effect on the responses. To obtain simultaneously the maximum efficiency and concentrate yield values, the operating conditions can be set at a time of 58 min, coolant temperature of – 5 ºC, and mechanical stirring of 1035 rpm. The results found in the PFC system suggest that the protein is easier to be occluded in the ice. A second stage for the freeze concentration process was proposed (by BFC), which in turn demonstrated to be an efficient method to concentrate skim lactose-free milk. In this system, the protein was well concentrated in the liquid phase. On the other hand, carbohydrates did not concentrate like proteins, but even so, a high concentration of this compound was found. Finally, concentrate 1 and 2 could be mixed and used for the development of new lactose-free dairy products.

ACKNOWLEDGMENTS

The authors are grateful to the Coordination of Improvement of Higher Education Personnel (CAPES, Brazil) [CAPES-PRINT, Project number 88887.310560/2018-00]; to National Council for Scientific and Technological Development (CNPq, Brazil) by the financial support [CNPq, 405965/2016-8]; and to Coordination of Improvement of Higher Education Personnel (CAPES, Brazil) by the scholarship [001]. The authors are also grateful to the Universitat Politecnica de Catalunya (Spain) for scientific support.

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CONCLUSÕES

No que diz respeito aos três primeiros capítulos da parte experimental (Capítulos 2, 3 e 4), devemos avaliar sob um contexto global, qual das microesferas produzidas seria a mais adequada para a produção industrial em larga escala. A princípio, se pode assumir que isso dependeria da aplicação principal do pó. Por exemplo: se o produto a ser vendido será submetido a longo armazenamento em temperatura ambiente, a indicação de uso é do Pó 2 (feito com leite sem lactose e inulina). Por sua vez, o Pó 3 (feito com leite sem lactose e oligofrutose) seria o mais adequado se o fator preponderante fosse a chegada do maior número de células viáveis no intestino delgado, associado a um armazenamento refrigerado. Além disso, é um material que suporta condições de umidade relativas maiores (até 43%). No caso de processamento composto de várias etapas, a utilização do Pó 1 seria mais vantajosa dada suas melhores propriedades de fluxo. Para a utilização deste pó, também seria ideal a manutenção da temperatura de armazenamento ao redor de 4 ºC, ou fixar a validade do produto em 30 dias. Se, porém, o âmbito geral tem que ser avaliado, e em consequência há de se escolher um único pó que atenda a todos os requisitos, o pó indicado é o Pó 2, dada sua alta estabilidade térmica e estabilidade ao armazenamento. O maior cuidado no seu uso seria a manutenção da umidade relativa a até 33%. Isso implicaria em atenção na escolha da embalagem do produto.

Ao estudar o Capítulo 5 (referente à crioconcentração do leite), este projeto experimental seria agraciado se houvesse investimento e, em consequência, testes em larga escala visando utilizar PFC e BFC concomitantemente. O leite concentrado poderia ser utilizado na sequência para a fabricação de iogurte concentrado, ou ainda em queijos e sorvetes. Além do mais, a venda do leite concentrado como simples acompanhamento para cafés é comum em países europeus e de outras regiões do globo.

CONSIDERAÇÕES FINAIS

O leite é uma excelente fonte de nutrientes e seus derivados são produtos igualmente nutritivos, ricos sensorialmente, e amplamente consumidos em diversas regiões do mundo. No entanto, um importante segmento da população mundial adulta sofre de intolerância à lactose. Esse quadro é causado pela redução ou perda da atividade da enzima intestinal lactase-florizina hidrolase, responsável pela digestão da lactose. Essa alteração resulta num aumento da carga osmótica no intestino delgado, assim como favorece a fermentação da lactose pela flora bacteriana, o que leva a uma alta produção de ácidos graxos de cadeia curta e gases. Isso é seguido pelo aparecimento de dor abdominal, diarreia e flatulência. Além disso, estudos apontam que indivíduos com intolerância à lactose têm um risco aumentado de desenvolver várias doenças extra-intestinais, incluindo câncer. Por sua vez, o mercado de lácteos sem lactose possibilita que essa parcela da população usufrua dos benefícios do consumo do leite sem sofrer as consequências negativas da ingestão da lactose. A inserção de microrganismos probióticos nesses produtos poderia potencializar seu valor e sua percepção por parte dos consumidores, dada a alegação de funcionalidade dos mesmos. Além disso, estudos recentes indicam que a viabilidade celular não é um requisito essencial para exercer benefícios à saúde (pós-bióticos), o que representa uma interessante oportunidade na área de produtos lácteos funcionais e uma área emergente para o desenvolvimento de produtos lácteos sem lactose. Do mesmo modo, nota-se uma carência de trabalhos utilizando substâncias prebióticas nos produtos lácteos sem lactose, embora novas substâncias funcionais estejam sendo avaliadas e aplicações sejam feitas. Assim, esta é uma área que pode ser mais investigada e tem potencial para aumentar a gama de produtos prebióticos disponíveis no mercado.

Várias técnicas podem ser aplicadas para encapsular bactérias probióticas visando a adição das mesmas em produtos alimentares. O método apropriado de encapsulação deve ser cuidadosamente escolhido com base na bactéria a ser encapsulada, nos agentes encapsulantes e na matriz onde as micropartículas serão aplicadas. Neste sentido, o uso da técnica de encapsulação por *spray drying* oferece vantagens quando comparada com outras técnicas, devido a sua facilidade de implementação em nível industrial (“scale-up”). Tendências futuras apontam para a busca de novos agentes encapsulantes para melhorar a eficiência do processo de encapsulação e a proteção das bactérias probióticas. Os maiores desafios estão na aplicação das micropartículas em complexas matrizes de alimentos. Desta forma, estudar o sinergismo entre os agentes encapsulantes e os probióticos pode aprimorar seus efeitos funcionais.

A caracterização das micropartículas probióticas é de suma importância para avaliar de diferentes maneiras a eficiência dos agentes encapsulantes no que diz respeito à proteção das bactérias. Essa caracterização também auxilia na escolha de novos materiais encapsulantes e materiais de embalagem, e na determinação de condições de armazenamento mais eficientes. Além disso, é um estudo que dá suporte ao uso das micropartículas em aplicações de larga escala, uma vez que a incorporação de probióticos é de grande interesse para a indústria de derivados lácteos.

Neste trabalho, a técnica de *spray drying* foi empregada eficientemente na microencapsulação de *Bifidobacterium* BB-12, utilizando leite sem lactose como agente encapsulante em todas as formulações. Ademais, a tecnologia de crioconcentração foi aplicada neste leite, a fim de investigar a influência dos parâmetros de processo em respostas como eficiência e rendimento. As concentrações finais de carboidrato e proteína do leite concentrado também foram estudadas. Assim, como sugestões para pesquisas futuras e o consequente enriquecimento deste trabalho, são listadas as seguintes recomendações:

- Incluir a etapa do intestino grosso na simulação da passagem microbiana no trato gastrointestinal; ou seja, as etapas do cólon ascendente, transverso e descendente poderiam ser avaliadas;
- Outras análises de caracterização dos pós seriam bem-vindas, como por exemplo, molhabilidade, dispersibilidade, e microscopia confocal de varredura a laser. Sugere-se também a determinação da temperatura de transição vítrea de todos os pós;
- Investigar a sobrevivência da bifidobacteria nos pós quando estes estiverem expostos às condições de umidade relativa estudadas neste trabalho (11%, 33%, 43%, e assim sucessivamente). Isso porque, avaliações mais abrangentes sobre a sobrevivência microbiana nas diferentes condições de armazenamento poderiam fornecer melhores informações sobre a otimização das três formulações estudadas;
- Avaliar as características sensoriais das formulações de iogurte concentrado probiótico sem lactose;
- Além do conteúdo de carboidratos e proteínas após o processo de crioconcentração, o teor de minerais também pode ser avaliado (por exemplo, cálcio, magnésio, zinco e fósforo);
- Investigar as características físicas dos concentrados obtidos, como por exemplo, viscosidade e condutividade térmica;

- Conduzir a crioconcentração em bloco assistida por vácuo como sendo o primeiro ciclo do processo.

ANEXO A – Trabalhos apresentados em eventos

VII Semana Acadêmica de Ciência e Tecnologia de Alimentos

"Ciência e Tecnologia de Alimentos: Passado, presente e futuro"

06 a 10 de agosto de 2018

Florianópolis, SC

Certificado de Apresentação Oral

Certificamos que **Silvani Verruck**, apresentou oralmente o trabalho intitulado como "**MICROENCAPSULAÇÃO DE Bifidobacterium BB-12 COM LEITE SEM LACTOSE E PREBIÓTICO: AVALIAÇÃO DAS PROPRIEDADES DE COR**", cujos autores são *Adriana Dantas, Silvani Verruck, Bruna M. Maran, Sofia G. Garcia e Elane S. Prudêncio*, no dia **08 de agosto de 2018**, durante a programação da VII Semana Acadêmica de Ciência e Tecnologia de Alimentos (VII SACTA), em Florianópolis/SC

Florianópolis, setembro de 2018.

XV Encontro Regional Sul de Ciência e Tecnologia de Alimentos

"Caminhos da produção de alimentos: Biodiversidade e Inovação"

CERTIFICADO

Certificamos que

MARIA HELENA MACHADO CANELLA, CALLEBE CAMELO-SILVA, SOFIA GRECHI GARCIA, EULÁLIA LOPES DA SILVA BARROS, ADRIANA DANTAS, ELANE SCHWINDEN PRUDENCIO

Participou(aram) da XV ERSCTA - Encontro Regional Sul de Ciência e Tecnologia de Alimentos, realizado no período de 28 e 29 de novembro de 2019, em Curitiba - PR.

Na qualidade de autor(es) do Trabalho Científico: AVALIAÇÃO DA CONCENTRAÇÃO DE PROTEÍNAS NO LEITE DE CABRA DESNATADO ATRAVÉS DOS PROCESSOS DE CRIOCONCENTRAÇÃO EM BLOCOS E NANOFILTRAÇÃO

Curitiba, 29 de Novembro de 2019.

Leomara Floriano Ribeiro
Coordenadora da Comissão Científica do XV ERSCTA

Agnes de Paula Scheer
Presidente do XV ERSCTA

Promoção

Organização

ANEXO B – Publicações

Journal of Functional Foods 52 (2019) 243–257

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Functionality of the components from goat's milk, recent advances for functional dairy products development and its implications on human health

Silvani Verruck[a], Adriana Dantas[b], Elane Schwinden Prudencio[a,b,*]

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ARTICLE INFO

Keywords:
Goat's milk
Goat products
Prebiotic
Probiotic
Functional food

ABSTRACT

The growing consumer interest in goat's milk and dairy products is related to nutritive values and positive health benefits attached to these products. Goat's milk is known for its lower allergenic potential and better digestibility, when compared to bovine milk, as well as the presence of health-promoting compounds. Therefore, goat milk can be used in the manufacture of a wide variety of products and can also be used as carrier for functional components, such as prebiotic substances or probiotic bacteria. Some in vivo studies have been carried out to explore its therapeutic potential and have shown excellent biological effects. However, probiotic/prebiotic re-

Food Research International 127 (2020) 108752

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Stability of bifidobacteria entrapped in goat's whey freeze concentrate and inulin as wall materials and powder properties

Gabriela Rodrigues de Liz[a], Silvani Verruck[a], Maria Helena Machado Canella[a], Adriana Dantas[b], Sofia Grechi Garcia[c], Bruna Marchesan Maran[c], Fabio Seigi Murakami[d], Elane Schwinden Prudencio[a,b,*]

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ARTICLE INFO

ABSTRACT

LWT - Food Science and Technology 124 (2020) 109133

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Optimization of goat milk vacuum-assisted block freeze concentration using response surface methodology and NaCl addition influence

Maria Helena Machado Canella[a], Adriana Dantas[b], Mónica Blanco[c], Mercè Raventós[d], Eduard Hernandez[d], Elane Schwinden Prudencio[a,b,*]

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ARTICLE INFO

Keywords:
Concentration
Caprine milk
Optimization
Sodium chloride
Vacuum thawed

ABSTRACT

Response Surface Methodology was applied to optimize the effects of freezing time, vacuum conditions, and time under vacuum regarding concentrated yield response, resulting from optimal parameters of the milk vacuum-assisted block freeze concentration process. Additionally, it was verified the NaCl influence, using different salt contents (0.5, 1, 1.5, and 2%) addition and freezing time of 1 day, vacuum equal to 10 kPa, and time under vacuum 60 min, in goat milk vacuum-assisted freeze concentration performance. The concentrate with 1.5 and 2% of NaCl addition showed the highest values for the total solids (35.06 and 36.21 g 100 g^{-1}) and protein contents (10.43 and 10.70 g 100 g^{-1}), while the concentrate without NaCl addition concentrated more lactose content (17.42 g 100 g^{-1}). The samples with 1.5 and 2% of NaCl addition reached parameters of the process more satisfactory with a concentrate yield of 85.79 and 92.14%, concentration percentage of 28 and 32%, and efficiency of process approximately of 90%. Finally, the best performance was observed when used 1.5 and 2% NaCl addition in the goat milk submitted to the vacuum-assisted freeze concentration process.

Food Research International 141 (2021) 110135

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Influence of guabiroba pulp (*campomanesia xanthocarpa* o. berg) added to fermented milk on probiotic survival under *in vitro* simulated gastrointestinal conditions

Amanda Alves Prestes[a], Silvani Verruck[b], Maryella Osório Vargas[b], Maria Helena Machado Canella[b], Callebe Camelo Silva[a], Eulália Lopes da Silva Barros[a], Adriana Dantas[a], Luan Valdomiro Alves de Oliveira[b], Bruna Marchesan Maran[c], Mailson Matos[d], Cristiane Vieira Helm[e], Elane Schwinden Prudencio[a,b,*]

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ARTICLE INFO

Keywords:
Campomanesia xanthocarpa O. Berg
Gastrointestinal steps
Bifidobacterium BB-12
Antioxidant activity
Phenolic content
Yogurt

ABSTRACT

In fermented milks inoculated with two thermophilic strains (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*), guabiroba pulp (*Campomanesia xanthocarpa* O. Berg) was added in different concentrations: 5% (I5 sample) and 10% (I10 sample), compared to a control sample, with no pulp addition. In these fermented milks, *Bifidobacterium* BB-12 was added and the samples were submitted to a progressive gastrointestinal simulation *in vitro*. The cells count was performed, including the survival rates for all the progressive steps of the simulated digestion. Total phenolic content (TPC) and antioxidant activity analysis by FRAP (Ferric Reducing Antioxidant Power) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were performed in all the gastrointestinal steps. Before and

International Journal of Food Science +Technology

International Journal of Food Science and Technology 2020

Original article

Lactose-free skim milk and prebiotics as carrier agents *of Bifidobacterium* BB-12 microencapsulation: physicochemical properties, survival during storage and *in vitro* gastrointestinal condition behaviour

Adriana Dantas,[1] Silvani Verruck,[2] Gabriela Rodrigues de Liz,[2] Eduard Hernandez[3] & Elane Schwinden Prudencio[1,2*]

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(Received 12 June 2020; Accepted in revised form 17 September 2020)

Summary *Bifidobacterium* BB-12 was microencapsulated by spray drying using lactose-free milk, lactose-free milk and inulin, and lactose-free milk and oligofructose, resulting in powders 1, 2 and 3, respectively. The highest encapsulation yield (88.01%) and the highest bifidobacteria viability during 120 days of storage were noted for spray-dried powder 2. Spray-dried powders 1 and 3 show a higher tendency to yellow colour. After being submitted to in vitro-simulated gastrointestinal conditions, the best probiotic survival rate result was found for spray-dried powder 3 (87.59%). Therefore, spray-dried powders containing prebiotics were the most appropriate combinations for microencapsulation of *Bifidobacterium* BB-12 and maintenance of cell viability during storage and gastrointestinal system, showing great potential to be used in lactose-free dairy products.

LWT - Food Science and Technology 151 (2021) 112175

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Current knowledge about physical properties of innovative probiotic spray-dried powders produced with lactose-free milk and prebiotics

Adriana Dantas [a], Silvani Verruck [b], Maria Helena Machado Canella [a], Bruna Marchesan Maran [a], Fabio Seigi Murakami [c], Lindiomar Borges de Avila Junior [d], Carlos Eduardo Maduro de Campos [d], Eduard Hernandez [e], Elane Schwinden Prudencio [a,b,*]

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ARTICLE INFO

ABSTRACT

Received: 30 May 2021 | Revised: 19 July 2021 | Accepted: 17 August 2021

DOI: 10.1111/jfpe.13867

ORIGINAL ARTICLE

Journal of Food Process Engineering WILEY

The combined use of progressive and block freeze concentration in lactose-free milk: Effect of process parameters and influence on the content of carbohydrates and proteins

Adriana Dantas[1] | Gisela J. Quinteros[2] | Shadi Yaribeigi Darvishvand[2] | Mónica Blanco[3] | Eduard Hernandez[2] | Elane Schwinden Prudencio[1] | Shafirah Samsuri[4]

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Inovações e Avanços em Ciência e Tecnologia de Leite e Derivados
Adriano Gomes da Cruz
Elane Schwinden Prudencio
Erick Almeida Esmerino
Tatiana Colombo Pimentel
Adriana Torres Silva e Alves
Leila Maria Spadoti
Patrícia Blumer Zacarchenco
Realização
Patrocinadores

Inovações no Desenvolvimento de Derivados Lácteos Probióticos, Prebióticos e Simbióticos

Adriana Dantas
Silvani Verruck
Celso Fasura Balthazar
Erick Almeida Esmerino
Jonas Toledo Guimarães
Ramon Silva Rocha
Tatiana Colombo Pimentel
Adriano Gomes da Cruz
Elane Schwinden Prudencio

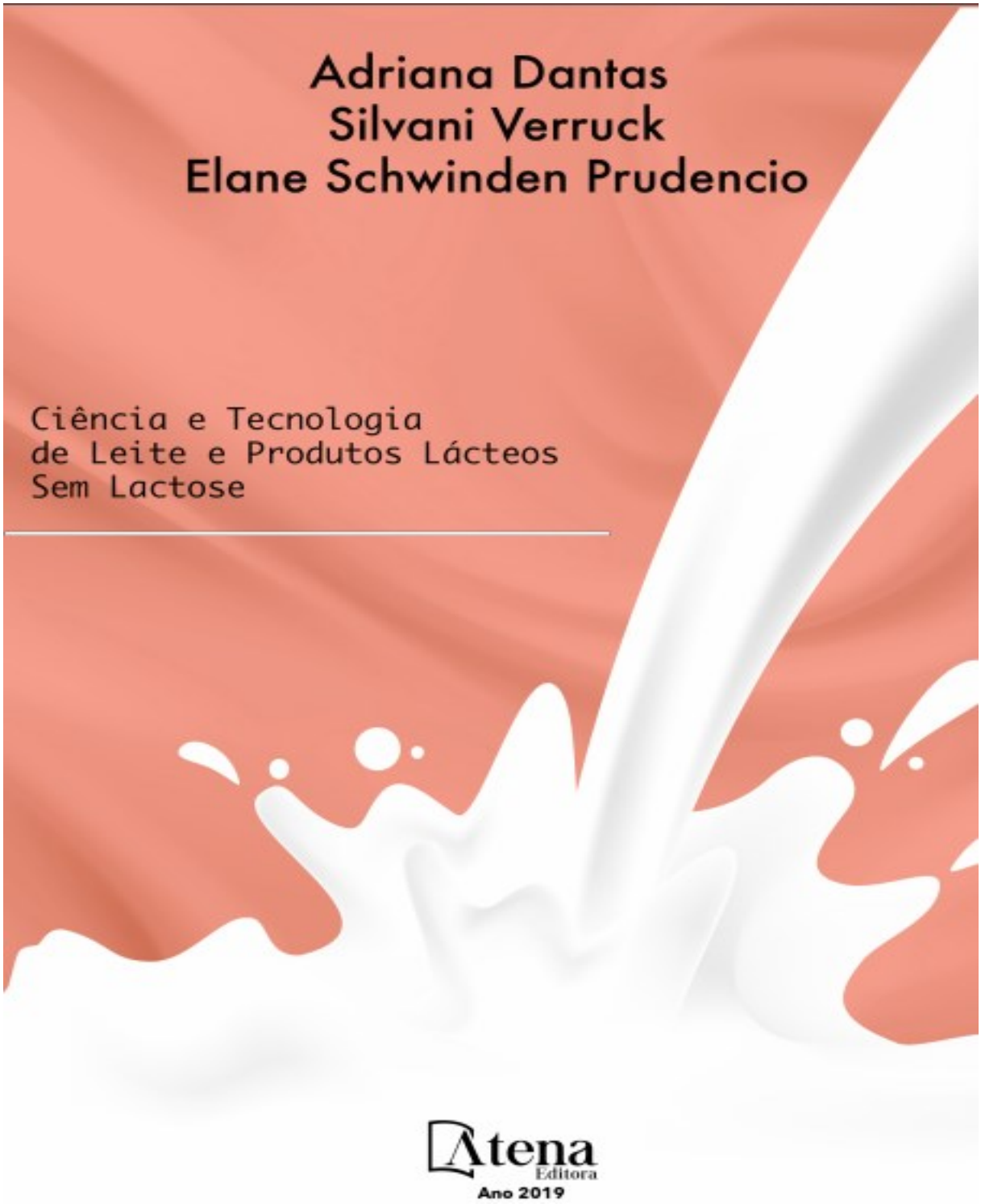
Conceitos apresentados neste capítulo

Este capítulo atualiza os conceitos existentes para microrganismos probióticos e substâncias prebióticas. As definições, funções e utilizações gerais dos probióticos foram abordadas, bem como os processos envolvidos em sua produção. Os prebióticos também tiveram suas definições, funções e utilizações gerais descritas. Além disso, atualizações no efeito da aplicação dos probióticos, prebióticos ou sua associação (simbióticos) em leite e em derivados lácteos foram relatadas.

10.1. INTRODUÇÃO

No Brasil, os alimentos com alegações de propriedades funcionais e ou de saúde são regulamentados pela Agência Nacional de Vigilância Sanitária. Os ingredientes fontes dos nutrientes ou não nutrientes relacionados à alegação de propriedade funcional ou de saúde devem ser comprovadamente seguros para consumo humano. Ao todo, são seis grandes grupos que compõem a lista de nutrientes e não nutrientes que tem alegações padronizadas e entre estes estão os microrganismos probióticos e alguns compostos alimentares com efeito prebiótico (ANVISA, 2002).

Por definição probióticos são microrganismos vivos capazes de melhorar o equilíbrio microbiano intestinal produzindo efeitos benéficos à saúde do indivíduo (ANVISA, 2002). Para probióticos, a alegação de propriedade funcional ou de saúde é avaliada caso a caso e deve ser proposta pela empresa fabricante. A avaliação será embasada nos requisitos estabelecidos pela Resolução n. 18/1999 (ANVISA, 1999).


Adriana Dantas
Silvani Verruck
Elane Schwinden Prudencio
Ciência e Tecnologia
de Leite e Produtos Lácteos
Sem Lactose
Atena
Editora
Ano 2019

Dados Internacionais de Catalogação na Publicação (CIP)
(eDOC BRASIL, Belo Horizonte/MG)

D192c

Dantas, Adriana.
Ciência e tecnologia de leite e produtos lácteos sem lactose [recurso eletrônico] / Adriana Dantas, Silvani Verruck, Elane Schwinden Prudencio. – Ponta Grossa (PR): Atena Editora, 2019.

Formato: PDF
Requisitos de sistema: Adobe Acrobat Reader
Modo de acesso: World Wide Web
Inclui bibliografia.
ISBN 978-85-7247-112-1
DOI 10.22533/at.ed.121191102

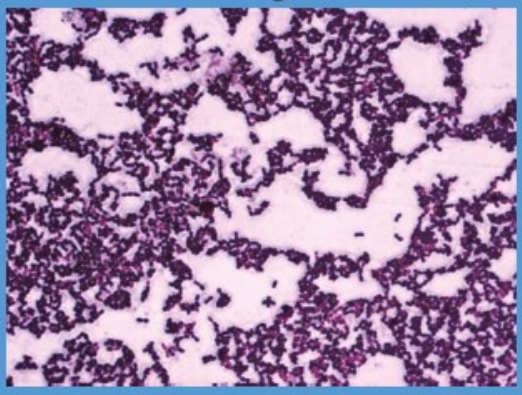
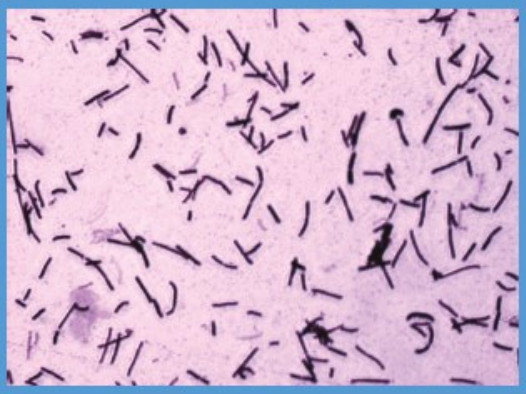
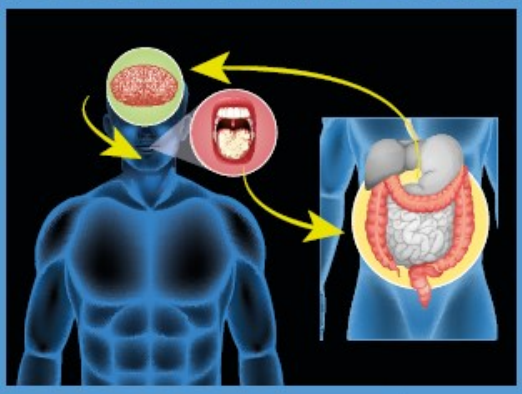
1. Engenharia de alimentos. 2. Laticínios – Processamento. 3.Leite – Análise. 4. Tecnologia de alimentos – Lactose. I. Verruck, Silvani. II. Prudencio, Elane Schwinden.

CDD 637

Elaborado por Maurício Amormino Júnior – CRB6/2422

Probióticos e Prebióticos

Desafios e Avanços

Adriano Gomes da Cruz
Adriana Torres Silva e Alves
Elane Schwinden Prudêncio
Erick Almeida Esmerino
Leila Maria Spadoti
Márcia Cristina Silva
Michel Reis Messora
Patrícia Blumer Zacarchenco
Tatiana Colombo Pimentel

25 anos
Setembro Editora

Realização
INSTITUTO FEDERAL Rio de Janeiro
ITAL
SÃO PAULO GOVERNO DO ESTADO
Indústria de Ingredientes e Alimentos

Instituições Participantes
INSTITUTO FEDERAL Farroupilha
Indústria de Laticínios

Patrocínio
Globalfood ADVANCED FOOD TECHNOLOGY

Leites e Derivados Probióticos e Prebióticos de Espécies Não Bovinas

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Amanda Alves Prestes
Silvani Verruck
Maria Helena Machado Canella
Elane Schwinden Prudêncio
Erick Almeida
Celso Fasura Balthazar
Ramon Silva Rocha
Marcia Cristina Silva
Adriano Gomes da Cruz

Resumo

Este capítulo engloba a utilização de leites de búfalas, cabras, ovelhas e de outras espécies como matrizes benéficas para o desenvolvimento de produtos probióticos. Na descrição das características dos leites, são enfatizadas suas composições no favorecimento da multiplicação de células probióticas como também seus aspectos contribuintes na finalidade prebiótica. Ao final, foram abordados estudos em destaque sobre o desenvolvimento de produtos lácteos, de cada espécie em particular, e seus resultados no beneficiamento seletivo de microrganismos probióticos.

1. Introdução

O leite de vaca é o leite mais consumido em todo o mundo, dominando a produção mundial de leite com 675 milhões de toneladas em 2017. Assim, como pode ser visualizado na Figura 1, em 2017 81,61% da produção mundial de leite é bovino, seguido por leites de outras espécies, como o de búfala (14,54%), cabra (2,25%), ovelha (1,26%) e de camela (0,34%) (FAOSTAT, 2020). Esses dados foram apresentados pela Organização das Nações Unidas para a Alimentação e a Agricultura (FAO) em 2019. No entanto, as fazendas produtoras de leite de espécies não bovinas representam uma parte significativa da economia agrária em muitos países onde a produção de vacas leiteiras não consegue ser explorada (CLARK; GARCÍA, 2017). O

Kefir e Kombucha: Alimentos Probióticos Emergentes

Silvani Verruck
Adriana Dantas
Amanda Alves Prestes
Maria Helena Machado Canella
Elane Schwinden Prudencio
Marcia Cristina da Silva
Erick Almeida Esmerino
Patrícia Blumer Zacarchenco
Adriano Gomes da Cruz

Resumo

Neste capítulo, serão tratados aspectos tecnológicos voltados ao processamento de *kefir* e *kombucha*. São apresentados os principais efeitos benéficos relacionados ao consumo desses produtos, bem como os principais microrganismos envolvidos na sua produção. É realizada também uma revisão sobre a metagenômica e as interações dos microrganismos com a matriz *kefir* e *kombucha*. Por fim, este capítulo aborda os principais conceitos envolvidos na produção de *kefir* e *kombucha* e sua perspectiva de utilização como produtos funcionais em escala industrial.

1. Introdução

Alimentos fermentados tradicionais, como o *kefir* e a *kombucha*, têm ganhado espaço na mesa dos consumidores. Os microrganismos probióticos vêm sendo selecionados naturalmente nesses produtos ao longo dos anos. Os efeitos dos probióticos nesses produtos estão sendo avaliados em um crescente número de estudos.

Para exemplificar como há crescimento do interesse do consumidor e, portanto, na produção desse segmento, o mercado de *kefir* foi estimado ao redor de 130 milhões de dólares americanos em 2014, enquanto, nos anos de 1990, ele era desprezível. Algumas indústrias importantes, mundialmente, no segmento de *kefir* apontadas pela Future Market Insights são: Lifeways Foods, Danone, Nourish Kefir, Babushka Kefir, Kenmare Living Foods, Happy Kombucha, Valio Eesti AS, Lifehouse Foods, Wallaby Yogurt Company, Best of Farms LLC, entre outros, (FUTURE MARKET INSIGHTS, 2019). No Brasil, as empresas Keiff e A Leiteria produzem *kefir*.

Bebidas Vegetais Probióticas e Prebióticas

Maria Helena Machado Canella
Elane Schwinden Prudencio
Adriana Dantas
Amanda Alves Prestes
Silvani Verruck
Erick Almeida Esmerino
Adriano Gomes da Cruz

Resumo

As bebidas, muitas vezes, não são consumidas pelo seu valor alimentar, mas sim como fonte de energia. Entretanto, as bebidas tornaram-se uma tendência alimentar, porque representam para todas as gerações de consumidores uma forma de dieta simplificada a fim de obter maior nutrição. Até o momento, a literatura relata vários exemplos de bebidas com a adição de diferentes nutrientes. Inúmeros objetivos e aspectos foram levados em consideração para garantir fortificações bem sucedidas, inclusive, aceitando novos desafios tecnológicos. Este capítulo fornecerá uma abordagem sobre o valor de prebióticos e/ou probióticos em bebidas de origem vegetal, englobando uma ampla gama de produtos, como bebidas à base de soja, cereais, vegetais, frutas, soja e frutas, e frutas e vegetais. Também serão abordados os principais tipos de prebióticos e probióticos presentes em bebidas vegetais, bem como as principais tecnologias envolvidas no desenvolvimento de bebidas vegetais adicionadas de prebióticos e/ou probióticos.

1. Introdução

O interesse do consumidor em nutrição e saúde contribuiu para o aumento no desenvolvimento de produtos classificados como probióticos, prebióticos e simbióticos. De acordo com Hill *et al.* (2014), probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro. A grande maioria dos trabalhos científicos classificaram como culturas probióticas espécies de *Lactobacillus* e *Bifidobacterium*. Com a recente reclassificação de espécies de microrganismos que pertenciam ao gênero *Lactobacillus*, contudo, há espécies probióticas realocadas em outros gêneros. Por exemplo, o *Lactobacillus casei* e o *Lactobacillus reuteri* estão, atualmente, reclassificados, respectivamente, como *Lacticaseibacillus casei* e *Limosilactobacillus reuteri*, pertencendo, portanto, a outros gêneros. Este capítulo manteve a nomenclatura de espécies que pertenciam ao gênero *Lactobacillus*, que passou por reclassificação publicada nos primeiros meses do ano de 2020. Estão também colocados os nomes das espécies após a reclassificação.