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MICROENCAPSULAÇÃO DE PROBIÓTICOS E EXTRATO BIOATIVO DE CASCA DE CEBOLA ROXA (*Allium cepa* L.) POR GELIFICAÇÃO IÔNICA EXTERNA

Santa Maria, RS,

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Tese apresentada ao Curso de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciência e Tecnologia dos Alimentos**.

Orientador: Prof. Dr. Cristiano Ragagnin de Menezes

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Greice Carine Raddatz

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Aprovado em 02 de fevereiro de 2022:

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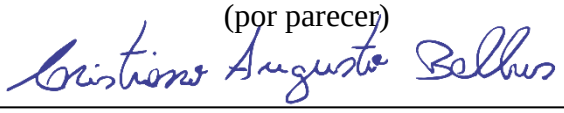

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Santa Maria, RS
2022

DEDICATÓRIA

A todos aqueles que, de alguma forma,incentivam e/ou contribuem com a pesquisa no Brasil!

RESUMO

MICROENCAPSULAÇÃO DE PROBIÓTICOS E EXTRATO BIOATIVO DE CASCA DE CEBOLA ROXA (*Allium cepa* L.) POR GELIFICAÇÃO IÔNICA EXTERNA

AUTORA: Greice Carine Raddatz
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O desenvolvimento de produtos contendo um ou mais compostos bioativos tem se tornado um meio atrativo ao consumidor como forma de estabelecer uma melhor qualidade de vida. Dentre os compostos bioativos, destacam-se os probióticos e os compostos antioxidantes, no entanto, sua aplicação em matrizes alimentares se torna limitada visto que são extremamente sensíveis a condições adversas. Sendo assim, novas alternativas para introduzir estes compostos ao cotidiano do consumidor são necessárias. A microencapsulação é uma tecnologia promissora, segura e eficaz para a entrega de diversos compostos bioativos. Dentre as diversas técnicas de microencapsulação, tem-se a gelificação iônica externa. O objetivo deste trabalho foi desenvolver micropartículas de alginato contendo o probiótico *Lactobacillus casei* LC03 em combinação com extrato de casca de cebola roxa *(Allium cepa* L.*)* em diferentes concentrações (5, 20 e 40%), por meio da técnica de gelificação iônica externa (Artigo II), para posterior aplicação em uma matriz alimentícia (Artigo III). No Artigo II foi avaliada a viabilidade dos probióticos sob condições gastrointestinais simuladas, durante armazenamento a -18, 7 e 25 °C por 90 dias e resistência das micropartículas ao tratamento térmico (72 °C / 15 seg e 63 °C / 30 min). Além da morfologia, diâmetro médio e eficiência de encapsulação das micropartículas. Por fim, no Artigo III, realizou-se a adição das micropartículas em polpa de morango, onde foram realizadas análises microbiológicas e físico-químicas da polpa, viabilidade gastrointestinal e vida útil dos probióticos no produto, além de tamanho, morfologia e eficiência de encapsulação das micropartículas. As micropartículas apresentaram variação de tamanho de 149.29 a 167.05 µm no Artigo II e de 136.00 a 305.00 µm no Artigo III. A eficiência de encapsulação dos probióticos e dos compostos presentes no extrato foi satisfatória em ambos os manuscritos. As micropartículas foram capazes de proteger os probióticos frente ao tratamento térmico nas diferentes temperaturas (Artigo II). No Artigo II, as micropartículas do tratamento contendo alginato + 20% de extrato apresentaram melhor sobrevivência do probiótico sob condições gastrointestinais simuladas, já ao se realizar a aplicação das diferentes micropartículas na polpa de morango, as formulações com alginato e alginato + 5% de extrato demonstraram resultados mais satisfatórios (Artigo III). Quanto a vida útil dos probióticos durante o armazenamento, no Artigo II, ao se avaliar as temperaturas de 25, 7 e -18 °C, todas as formulações de micropartículas se mantiveram viáveis até o final do experimento (90 dias) em -18 °C, o que justifica a escolha da polpa de morango congelada para a aplicação das micropartículas. Quando aplicadas na polpa (Artigo III), os tratamentos contendo alginato e alginato + 5% de extrato alcançaram o maior período de viabilidade (60 dias). Desta forma, as micropartículas desenvolvidas nesse estudo se mostram viáveis para o desenvolvimento de uma nova matriz funcional e vegana, permitindo aumentar a diversificação de produtos que contenham probióticos.

Palavras-chave: microencapsulação, *Lactobacillus casei, Allium cepa L.*, compostos bioativos.

ABSTRACT

MICROENCAPSULATION OF PROBIOTICS AND BIOACTIVE EXTRACT FROM BLACK ONION (*Allium cepa* L.) PEEL BY EXTERNAL IONIC GELATION

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The development of products containing one or more bioactive compounds has become an attractive way to the consumer as a way to establish a better quality of life. Among the bioactive compounds, probiotics and antioxidant compounds stand out, however, their application in food matrices becomes limited as they are extremely sensitive to adverse conditions. Therefore, new alternatives to introduce these compounds to the consumer's daily life are needed. Microencapsulation is a promising, safe and effective technology for the delivery of several bioactive compounds. Among the various microencapsulation techniques, there is the external ionic gelation. The objective of this work was to develop alginate microparticles containing the probiotic *Lactobacillus casei* LC03 in combination with red onion (*Allium cepa* L.) peel extract at different concentrations (5, 20 and 40%), using the external ionic gelation technique (Article II), for later application in a food matrix (Article III). Article II evaluated the viability of probiotics under simulated gastrointestinal conditions, during storage at -18, 7 and 25 °C for 90 days and resistance of microparticles to heat treatment (72 °C / 15 sec and 63 °C / 30 min). In addition to the morphology, average diameter and encapsulation efficiency of microparticles. Finally, in Article III, microparticles were added to strawberry pulp, where microbiological and physicochemical analyzes of the pulp, gastrointestinal viability and shelf life of the probiotics in the product were performed, as well as size, morphology and encapsulation efficiency of the microparticles. The microparticles varied in size from 149.29 t to 167.05 µm in Article II and from 136.00 to 305.00 µm in Article III. The encapsulation efficiency of probiotics and compounds present in the extract was satisfactory in both manuscripts. The microparticles were able to protect the probiotics against heat treatment at different temperatures (Article II). In Article II, the microparticles of the treatment containing alginate + 20% extract showed better survival of the probiotic under simulated gastrointestinal conditions, as the different microparticles were applied to the strawberry pulp, the formulations with alginate and alginate + 5% extract demonstrated more satisfactory results (Article III). As for the shelf life of probiotics during storage, in Article II, when evaluating temperatures of 25, 7 and -18 °C, all microparticle formulations remained viable until the end of the experiment (90 days) at -18 °C C, which justifies the choice of frozen strawberry pulp for the application of microparticles. When applied to the pulp (Article III), the treatments containing alginate and alginate + 5% extract reached the longest period of viability (60 days). Thus, the microparticles developed in this study are viable for the development of a new functional and vegan matrix, allowing to increase the diversification of products that contain probiotics.

Keywords: microencapsulation, *Lactobacillus casei, Allium cepa L,* bioactive compounds

SUMÁRIO

APRESENTAÇÃO

Esta tese segue as normas estabelecidas na Estrutura e Apresentação de Monografias, Dissertações e Teses – MDT da UFSM (UFSM 2021). Os resultados estão apresentados na forma de um capítulo de livro e três artigos, sendo o Artigo I de revisão bibliográfica, e o Artigo II e Artigo III com resultados da pesquisa. Ao final dessa tese, encontram-se os itens DISCUSSÃO GERAL e CONCLUSÕES, apresentando uma compilação de interpretações e comentários a respeito dos resultados demonstrados nos artigos científicos.

1. INTRODUÇÃO

Devido à conscientização acerca dos benefícios que hábitos de vida saudáveis geram ao nosso organismo, tem se percebido um aumento na busca pelo consumo de produtos que forneçam não apenas uma nutrição básica, mas também, compostos capazes de reduzir o risco de doenças e aumentar o bem-estar, repercutindo em benefícios a longo prazo. Como consequência disso, compostos como antioxidantes e probióticos despertam o interesse da indústria alimentícia para o aumento de pesquisas neste campo, que repercutem no desenvolvimento de novos produtos funcionais com um alto potencial comercial.

Os probióticos são caracterizados como microrganismos vivos que conferem efeitos positivos ao hospedeiro quando ingeridos em quantidades adequadas (HILL et al., 2014). Estes microrganismos, dentre outros diversos benefícios, atuam melhorando a saúde intestinal, quadros de intolerância à lactose além de reduzirem o risco de inúmeras doenças crônicas, degenerativas e cardiovasculares (KECHAGIA et al. 2013).

Já os compostos antioxidantes, onde se enquadram compostos fenólicos, pigmentos, flavonoides, vitaminas, antocianinas, dentre outros, são elementos funcionais capazes de atuar frente a reações oxidativas, podendo inibi-las ou neutraliza-las (LORENZO et al., 2017; SAMUZ et al., 2018). A cebola é um produto amplamente cultivado e consumido no mundo todo e contém elevado teor de polifenóis, com alta atividade antioxidante e anti-inflamatória (COZZOLINO et al., 2021, SAVIANO et al., 2019).

Um fator limitante para a administração destes compostos, no entanto, é a preservação da sua viabilidade e estabilidade para que atinjam seu local de ação, exercendo suas funções de saúde para o hospedeiro, uma vez que podem ser prejudicados pela luz, oxigênio, temperatura, umidade, sais biliares, baixo pH, dentre outros.

Processos como a microencapsulação permitem a proteção, o isolamento ou a liberação controlada de determinadas substâncias, incluindo compostos bioativos. Além disso, a microencapsulação surge como uma alternativa para unir diferentes compostos bioativos em uma única matriz, aumentando os benefícios à saúde obtidos através do seu consumo, além de possibilitar efeitos sinérgicos entre estes diferentes compostos, aumentando sua vida útil e biodisponibilidade. Desta forma, o desenvolvimento de novos alimentos funcionais por meio do uso da microencapsulação além de permitir o aumento da sua estabilidade, vem como uma alternativa aos adeptos do veganismo, bem como ao aumento de consumidores com intolerância à lactose e alergias às proteínas do leite (FURTADO MARTINS et al., 2013),

além de fornecer ao público consumidor, uma gama maior de opções para a sua administração diária.

O Brasil é um país com grande diversidade de frutas, que são capazes de auxiliar na redução do risco de diversas doenças. Segundo a Organização Mundial da Saúde, a baixa ingestão de frutas e vegetais é um dos principais fatores de causa de doenças no mundo inteiro (OMS, 2002). O desenvolvimento de alimentos funcionais a base de frutas é interessante do ponto de vista nutricional, além de garantir novas opções para os mais diversos consumidores.

Diante do exposto, ressalta-se a importância do presente trabalho no sentido explorar a tecnologia de microencapsulação, a partir da técnica de gelificação iônica externa, como forma de unir as propriedades funcionais do probiótico *Lactobacillus casei* e do extrato de casca de cebola roxa (*Allium cepa L.*) em micropartículas de alginato, até então, não estudados concomitantemente, a fim de se obter um sistema que garanta a viabilidade e funcionalidade probiótica estendida.

Em um segundo momento, selecionar o melhor tratamento das micropartículas e introduzi-las em polpa de morango congelada, bem como observar o comportamento das micropartículas probióticas no produto.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a estabilidade do probiótico *L. casei* LC03 microencapsulado juntamente com extrato de casca de cebola roxa, quando comparados ao tratamento controle (sem extrato), ambos produzidos pela técnica de gelificação iônica externa e avaliar a sua aplicação em polpa de morango congelada.

2.2 OBJETIVOS ESPECÍFICOS

- A partir da casca de cebola roxa, obter o seu extrato pelo método convencional com etanol e caracterizá-lo quanto a sua atividade antioxidante, teor de flavonoides, antocianinas monoméricas totais e compostos redutores;
- Desenvolver micropartículas contendo probióticos e extrato bioativo por gelificação iônica externa;
- Avaliar a influência do extrato de casca de cebola roxa em diferentes concentrações (5%, 20% e 40%) na viabilidade das micropartículas probióticas;
- Avaliar a distribuição de tamanho das micropartículas e a eficiência de encapsulação probiótica e do extrato;
- Avaliar a estabilidade dos probióticos microencapsulados frente a condições que simulem o ambiente gastrointestinal;
- Avaliar a resistência e a viabilidade das micropartículas frente ao tratamento térmico (72 °C / 15 seg e 63 °C / 30 min);
- Avaliar a vida útil das micropartículas em diferentes condições de temperaturas (-18 ° C, 7 ° C e 25 °C) por 90 dias de armazenamento;
- Selecionar as micropartículas com melhor desempenho para aplicação em polpa de morango;
- Avaliar as características físico-químicas e microbiológicas da polpa;
- Avaliar a viabilidade dos probióticos nas micropartículas após aplicação na polpa;
- Avaliar a vida útil e sobrevivência a condições gastrointestinais simuladas dos probióticos microencapsulados na polpa.

3. REVISÃO BIBLIOGRÁFICA

3.1 ALIMENTOS FUNCIONAIS

De acordo com a definição, os alimentos funcionais além de exercerem seu papel nutricional como parte da dieta normal do consumidor, ainda demonstram benefícios à saúde, podendo contribuir na diminuição do risco de doenças crônicas, incluindo o câncer, doenças cardiovasculares, dentre outras (IRAPORDA et al., 2019).

A ANVISA- Agência Nacional de Vigilância Sanitária- é a regulamentadora brasileira de alimentos funcionais, através das resoluções RDC n° 18 e 19 datadas de abril de 1999, da RDC n° 2 datada de janeiro de 2002 e da lista de alimentos com alegações de propriedades funcionais e ou de saúde, novos alimentos/ingredientes, substâncias bioativas e probióticas aprovada em julho de 2008 (ANVISA, 1999a, 1999b, 2002, 2008).

CASTRO (2003) estabeleceu 3 critérios para a classificação de um alimento como sendo funcional de acordo com a definição proposta pela ANVISA,

-a) deve ser obtido a partir de fontes naturais, ou seja, não ser apresentado em cápsulas, comprimidos ou suplementos;

-b) ter seu consumo como parte da dieta diária; e

-c) resultar em um efeito específico quando ingerido, para a regulação de um processo metabólico, como: aumento dos mecanismos de defesa biológica, prevenção de doenças, aumento da resistência, controle das condições físicas naturais ou envelhecimento e outros.

Os principais compostos caracterizados como funcionais são os probióticos, prebióticos, fibras alimentares, vitaminas antioxidantes, como a vitamina E, os carotenoides, flavonoides, ácidos graxos poli-insaturados ômega 3 e 6, dentre outros (MORAES; COLLA 2006).

Apesar do aumento nas pesquisas para o desenvolvimento de alimentos funcionais, poucos são os produtos encontrados a disposição do consumidor atualmente. O consumo de probióticos, por exemplo, em sua maioria se dá através de produtos lácteos, porém a desvantagem dos laticínios inclui intolerância à lactose, teor de colesterol, requerem refrigeração e possuem vida útil relativamente curta (LALICIC-PETRONIJEVIC et al., 2017).

3.2 PROBIÓTICOS

Os probióticos são “microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro” (FAO / WHO, 2006). Dentre os efeitos fisiológicos benéficos atribuídos à ingestão de probióticos estão a redução do pH no intestino com consequente inibição do desenvolvimento de microrganismos patogênicos, melhora da absorção de cálcio (PANDEY; NAIK; VAKIL, 2015), controle de distúrbios gastrointestinais, como constipação, diarréia ou síndrome do intestino irritável, além de atuarem na prevenção de diversos tipos de câncer, na redução do colesterol e controle glicêmico (KECHAGIA et al., 2013; MOHAMADSHAHI et al., 2014; ZARFESHANI et al., 2011) e depressão (FOSTER et al., 2013).

Apesar de serem altamente benéficos, para que os probióticos possam resultar em efeitos benéficos para o hospedeiro, eles devem ser aceitos como parte diária da dieta do consumidor, visto que existe uma recomendação diária do seu consumo. Um alimento vendido com alegações probióticas, deve conter um número de células viáveis de culturas probióticas de, pelo menos, 10^6 a 10^7 UFC g^{-1} (FAO/OMS, 2001).

Os microrganismos pertencentem ao gênero dos lactobacilos, como *L. acidophilus, L. rhamnosus, L. paracasei, L. plantarum,* dentre outros, são as bactérias mais comuns e utilizados para fins probióticos (SAAD et al., 2013).

3.2.1 Gênero *Lactobacillus*

Os lactobacilos são caracterizados como Gram-positivos, incapazes de formar esporos, com forma bacilar ou cocobacilar e ausência de flagelos, aerotolerantes ou anaeróbios e estritamente fermentativos, produzindo ácido lático (homofermentativo) ou quantidades equimolares de ácido lático e outros compostos (heterofermentativos) (SOCCOL et al., 2010). Estes microrganismos constituem somente 1% da microbiota fecal e possuem faixa de crescimento ótima em pH entre 5,5 e 6,3 (ANTUNES et al., 2007;GIONCHETTI et al., 2005).

O *Lactobacillus casei* constitui uma fração substancial da microbiota de lactobacilos existente na mucosa intestinal humana e seu efeito ao hospedeiro inclui dentre outros, a proteção da mucosa intestinal frente a agentes infecciosos em consequência da diminuição da colonização de patógenos no intestino (CANO; PERDIGÓN, 2003),

A espécie *L. casei* desperta cada vez mais o interesse no setor comercial, visto que seus efeitos benéficos à saúde já foram documentados (CHANDRAN et al., 2013, FERNANDES; SHAHANI; AMER, 1987).

3.3 ANTIOXIDANTES

Os antioxidantes são uma alternativa contra os efeitos oxidativos dos radicais livres, atuando na redução do estresse oxidativo no corpo (OROIAN; ESCRICHE, 2015). Estes compostos doam átomos de hidrogênio aos radicais livres que causam oxidação, sendo o íon oxidado estável, incapaz de propagar a reação de oxidação (BUCK, 1981). Quimicamente, são definidos como compostos aromáticos, visto que apresentam ao menos uma hidroxila em sua estrutura (OLIVEIRA et al., 2007).

Alimentos que apresentem capacidade antioxidante reforçam o interesse dos pesquisadores como um meio natural de substituir o uso de antioxidantes sintéticos como o BHT (butil-hidroxi-tolueno) e BHA (butil-hidroxi-anisol), que, de acordo com estudos, demonstraram possíveis efeitos carcinogênicos, podendo apresentar riscos para a saúde do consumidor (GALVÃO et al., 2008, LORENZO et al. 2013, SOOBRATTEE et al., 2005).

Os antioxidantes naturais incluem os tocoferóis, vitamina C, carotenóides e compostos fenólicos e podem ser obtidos através de diversas fontes como vegetais, frutas, grãos, dentre outros, além de poderem ser isolados de diferentes partes do vegetal como nas sementes, casca, bagas, folhas e raízes (SUCUPIRA et al., 2012). Nesse contexto, substâncias concentradas extraídas de matérias-primas vegetais através do uso de algum solvente, denominadas por extratos, podem ser uma boa alternativa para a implementação de antioxidantes naturais em diferentes matrizes alimentícias.

No entanto, dificuldades podem ser encontradas quanto a manutenção da estabilidade e biodisponibilidade destes compostos, devido à sua instabilidade. Em contrapartida a isso, o uso de técnicas de encapsulação pode mascarar certas características sensoriais indesejáveis do extrato, ao mesmo tempo em que fornece proteção, prolonga sua meia-vida e permite a liberação controlada do composto (CALDERÓN-OLIVER et al, 2017).

3.4 CASCA DE CEBOLA ROXA

Pertencente à família Alliaceae, a cebola (*Allium cepa* L.), resulta do cruzamento das famílias Liliaceae e Amaryllidaceae (BREWSTER, 2008; BOITEUX; MELO, 2004; FRITSCH; FRIESEN, 2002). Com enorme difusão em quase todo o planeta, a cebola apresenta grande importância social e econômica, fazendo parte da alimentação diária de povos com características étnicas e culturais distintas (EMATER, 2007). Sua coloração e intensidade da cor são variáveis de acordo com o cultivar: brancas, verdes, amarelas,

marrom/vermelhas e roxas, sendo isto resultante da presença de determinados flavonoides (BREWSTER, 2008).

A cebola é considerada a fonte alimentar com maior quantidade de flavonoides (SLIMESTAD; FOSSEN; VAGEN, 2007), Ainda, o teor destes compostos apresentam variações de acordo com cada parte da planta, sendo que, a parte externa da cebola (casca) apresenta concentrações mais elevadas de flavonoides em comparação com a parte interna (polpa), podendo ser até 48 vezes maior (2-10 g / kg) (SUH et al., 1999). Destacam-se entre os flavonoides presentes neste alimento as quercetinas livres, quercetinas ligadas glicosidicamente e os produtos derivados da oxidação da quercetina (como os flavonóis menores e os compostos fenólicos), (COOK; SAMMAN, 1996; GULSEN; MAKRAS; KEFALA, 2007; LEE; JUNG; KIM, 2012; RAMOS et al., 2006; SINGH et al., 2009). Além disso, encontram-se em elevada quantidade na cebola roxa as antocianinas, que são responsáveis pela pigmentação característica dessa variedade de cebola. Os flavonoides presente nas cascas de cebolas também possuem elevada capacidade antioxidante, podendo retardar reações de oxidação de certos compostos com o aumento de sua vida útil, além de possuir propriedades anticarcinogênicas (ALBISHI et al., 2013; SHIM et al., 2012).

Apesar de apresentar diversos aspectos benéficos e funcionais, devido a sua ampla produção e consumo, a cebola gera uma enorme quantidade de resíduos, que incluem principalmente sua casca, tanto a camada externa quanto as camadas mais internas. Desta forma, a elaboração de extratos bioativos a partir da casca de cebola roxa triturada (Figura 1) além de contribuir na diminuição dos resíduos provenientes deste alimento, seria uma fonte a contribuir significativamente para complementar as quantidades necessárias de flavonoides na dieta humana. Ainda, no entanto, o seu sabor picante e odor característico, associado à baixa estabilidade dos seus componentes bioativos frente a luminosidade, altas temperaturas e mudança de pH (MUNIN; EDWARDS-LÉVY, 2011), limitam a aplicação direta deste produto em alimentos, o que justifica a necessidade do uso de uma metodologia como a microencapsulação.

Figura 1 - Casca de cebola roxa seca e triturada

Fonte: acervo pessoal do autor (2020)

3.5 MICROENCAPSULAÇÃO E CO-ENCAPSULAÇÃO

O processo de encapsulação consiste no aprisionamento de um material ativo (aromas, óleos essenciais, microrganismos probióticos, compostos antioxidantes, vitaminas, enzimas, dentre outros) em um material de revestimento natural ou sintético, formando partículas que variam entre micrômetros até milímetros de tamanho (STĂNCIUC et al., 2018).

Esta técnica tem como finalidade básica garantir a proteção do material ativo contra condições desfavoráveis de armazenamento, do alimento em que são inseridos e de seu processamento, bem como frente à passagem pelo trato gastrintestinal, permitindo uma vida útil estendida, com maior estabilidade do composto ativo (WÜRTH et al., 2015). Além disso, a microencapsulação permite promover a liberação controlada do material ativo em seu local de ação, por meio de diferentes mecanismos, como, alteração de pH, força mecânica, temperatura, pela ação de enzimas, tempo, entre outros (COOK et al., 2012).

Dentre as diversas técnicas de encapsulação existentes, destacam-se em estudos: spray drying, spray chilling, leito fluidizado, gelificação iônica interna e externa e coacervação complexa (FAVARO-TRINDADE, 2011; ROKKA; RANTAMÄKI, 2010). A escolha do método de microencapsulação é dependente de fatores como o tamanho de micropartícula que se objetiva produzir, das propriedades físico-químicas do material ativo e de revestimento, do produto no qual se deseja aplicar as partículas, do mecanismo de liberação desejado, do custo e rendimento de produção, dentre outros (FAVARO-TRINDADE; PINHO, 2008).

A partir das diferentes técnicas de microencapsulação, também é possível unir dois ou mais compostos bioativos em uma única matriz, esta metodologia denomina-se por co-encapsulação (CHEN et al., 2013).

3.5.1 Gelificação iônica externa

A gelificação iônica permite a elaboração de micropartículas através do uso de íons multivalentes como $Ca2^{+}$, que ocasionam a reticulação de polieletrólitos (LUPO et al., 2015). A técnica consiste na mistura completa do material a ser encapsulado com o material de parede (solução polimérica), o qual é projetado através de um bico em uma solução que acarreta na gelificação do material de parede (por exemplo cloreto de cálcio) (CAVALHEIRO et al., 2015) (Figura 2).

Figura 2 - Produção de micropartículas por gelificação iônica externa

Fonte: acervo pessoal do autor (2020)

Esta técnica resulta em partículas com formato esférico além de uma variação em seu tamanho desde frações de mícron até alguns milímetros, dependendo dos materiais e métodos selecionados na produção das partículas (PAGANI et al., 2014).

A gelificação iônica permite o uso de diferentes hidrocolóides, tanto de origem vegetal, animal, como microbiana. Hidrocolóides são polímeros de polissacarídeos capazes de se dissolver ou dispersar em água resultando em uma solução de alta viscosidade (LEON et al., 2009; SANTOS, 2012).

Esta técnica resulta em uma alta eficiência de encapsulação do compostos ativo, além de permitir sua vida útil estendida visto que, se trata de uma técnica simples e branda em que não se aplicam temperaturas elevadas e nem solventes orgânicos.

3.6 MATERIAL ENCAPSULANTE

A seleção do material de parede implica nas características das micropartículas a serem produzidas. Para a sua seleção é necessário considerar as propriedades físico-químicas do material encapsulado, da técnica de microencapsulação, da não reatividade com o composto ativo durante o seu armazenamento, do sistema de liberação e da aplicação desejada (SUAVE et al., 2006).

O material de parede selecionado para produzir a micropartícula deve atuar como uma barreira de proteção frente a compostos orgânicos, além de possibilitar a conservação e manutenção da viabilidade do agente ativo contra condições adversas, como alta atividade de água, oxigênio, meios ácidos, dentre outros (AZEREDO, 2005; WEINBRECK; BODNÁR; MARCO, 2010).

Para a produção de micropartículas utilizam-se comumente lipídios (cera, parafina, triestearina, ácido esteárico, monoglicerídeos/ diglicerídeos, óleos e gorduras hidrogenadas), carboidratos (amidos, dextrinas, xarope de milho, celuloses, goma arábica, alginato e carragena), proteínas (glúten, caseína, gelatina e albumina e alguns polímeros sintéticos (poli D, l-ácido láctico, poliacrilatos, copolímeros de polietileno co-propileno e poli ε-caprolactona) (SUAVE et al., 2006; MARTINS et al, 2014), com destaque para compostos como alginato, gomas e amidos para o aprisionamento de células probióticas (TRIPATHI; GIRI, 2014).

3.7 POLPA DE MORANGO

A origem do morango se deu no norte da América e no Chile (REICHERT; MADAIL, 2003). O fruto atualmente cultivado (*Fragaria* x *ananassa*) surgiu pela hibridização espontânea de duas espécies (*F. chiloenses Mill*. e *F. virginiana* Duch), quando cultivadas lado a lado, resultando na geração de frutos maiores e de coloração mais avermelhada, muito distintos dos que eram produzidos anteriormente (FINN et al., 2013).

O sistema de produção do morangueiro é anual e a planta pode ser rasteira ou alcançar 15-30 cm de altura, com desenvolvimento mais propício em climas frios (FILGUEIRA, 2007), no entanto, o desenvolvimento de novos cultivares atualmente, permite uma maior distribuição dessa planta em ambientes e temperaturas diversificados (HENRIQUE; CEREDA, 1999).

No Brasil, o cultivo de morango se espalha pelo Sul, Sudeste, Centro-Oeste e Nordeste, sendo que no Sul e Sudeste está concentrada a maior produção (90 % da área cultivada no País) (PILLON, 2012). Visto que o cultivo e a colheita desse fruto requerem grande quantidade de mão de obra, a produção de morango gera renda e empregos, principalmente no caso de pequenos produtores rurais. A nível mundial, a produção de morangos também cresce exponencialmente, com alta de 7.879.108 toneladas em 2013 para 12.106.585 toneladas em 2019.

A busca crescente por uma alimentação mais saudável explica o aumento no consumo de frutas no mundo todo. O morango é rico em minerais, vinaminas (tiamina, riboflavina, niacina, vitamina B6, vitamina K, vitamina A, vitamina E e Vitamina C), aminoácidos, fibras e compostos fenólicos, como, por exemplo, flavonoides e antocianinas, além de possuir baixo teor calórico (100g = 32 kcal) (GIAMPIERI et al., 2012). Apesar disso, o morango é um fruto frágil, o que dificulta a sua colheita, transporte, armazenamento e comercialização, além de possuir alta atividade respiratória e microbiana, permanecendo em boas condições para consumo somente por um período entre 5 e 7 dias (NASCIMENTO; SILVA, 2010).

Para o aproveitamento integral de frutas como o morango, a fim de se evitar as perdas pós-colheita, a elaboração de polpas se torna um meio favorável (DANTAS et al., 2012). A polpa permite que ocorra o processamento na época de maior safra, com armazenamento por longos períodos, para que seja consumida o ano todo nas mais diversas regiões (BUENO, 2002; DANTAS et al., 2010).

4. MATERIAL E MÉTODOS

4.1 PREPARO DO INÓCULO

1 g da cultura probiótica *L. casei* LC 03 (Coana- Brasil) foi ativado em 100 mL de caldo MRS (Himedia) e incubado anaerobicamente por 15 a 18 h a 37 ± 1 °C. Em seguida, centrifugado a 4670 × g por 15 min a 4 ± 1 ºC e lavado com solução salina (0,85 %).

4.2 OBTENÇÃO DO EXTRATO DA CASCA DE CEBOLA ROXA

Os extratos foram preparados seguindo a metodologia proposta por VIERA et al., (2017) com pequenas adaptações. As amostras adquiridas em comércio local (Santa Maria-RS) foram higienizadas com hipoclorito de sódio (200 mg L^{-1}/ 20 min). Após, as cascas foram removidas e secas em forno com circulação de ar forçado (Marconi, MA-035/100, Piracicaba, Brasil) a 50 ± 1 °C por 24 h. Em seguida, as cascas foram trituradas em moinho de facas (Mill MA 630/1-Marconi) (velocidade = 5 rpm, tempo = 10 seg). Para a obtenção dos extratos, 3 g da amostra de casca de cebola roxa juntamente com 60 mL de etanol 80 % foram mantidos sob agitação a 80 rpm com o auxílio de um agitador mecânico por 120 min a 25 ± 2 °C. Os extratos foram então rotaevaporados e seu volume corrigido com água destilada para posterior utilização.

4.3 DETERMINAÇÃO DA CAPACIDADE REDUTORA

A capacidade redutora foi avaliada pelo método Folin-Ciocalteu, de acordo com ROESLER et al., (2007). As absorbâncias obtidas na reação foram lidas em espectrofotômetro no comprimento de onda de 765 nm e o teor de compostos redutores foi expresso em miligramas de ácido gálico / g de amostra seca (mg GAE g^{-1}).

4.4 DETERMINAÇÃO DO TEOR DE FLAVONOIDES

O conteúdo total de flavonoides foi avaliado de acordo com ZHISHEN; MENGCHENG; JIANMING, (1999). As absorbâncias obtidas na reação foram lidas em espectrofotômetro no comprimento de onda de 510 nm e o teor de flavonoides foi expresso em mg equivalente de quercetina (QE) por g de amostra seca (mg QE / g).

4.5 DETERMINAÇÃO DO TEOR DE ANTOCIANINAS MONOMÉRICAS TOTAIS

O conteúdo de antocianinas monoméricas totais foi realizado de acordo com GIUSTI; WROLSTAD, (2001), através do método de pH diferencial. As absorbâncias obtidas na reação foram lidas nos comprimentos de onda de 520 e 700 nm e o teor de antocianinas monoméricas totais foi calculado usando a seguinte equação:

Conteúdo de antocianina (mg / 100 g de matéria seca) = A x MW x DF ⁒ (ε x W)

Onde A é a absorbância (A520nm - A700nm) pH1,0 - (A520nm - A700nm) pH4,5, MW é o peso molecular da cianidina-3glucosídeo (C15H11O6, 449,2), DF é o fator de diluição, ε é a absortividade molar (26900), e W é o peso da amostra (g).

4.6 DETERMINAÇÃO DA CAPACIDADE ANTIOXIDANTE

A capacidade de absorbância do radical oxigênio (ORAC) foi analisada conforme proposto por OU et al. (2001), e os resultados foram expressos em μmol equivalentes de Trolox por grama de casca de cebola roxa (μmol de Trolox / g).

4.7 PRODUÇÃO DAS MICROPARTÍCULAS - TÉCNICA DE GELIFICAÇÃO IÔNICA EXTERNA CONTENDO *L. casei* E EXTRATO DE CASCA DE CEBOLA ROXA

A microencapsulação por gelificação iônica externa foi feita de acordo com a metodologia proposta por ETCHEPARE et al. (2016) com as adaptações. Para isso, 100 ml de uma mistura de 2 % de alginato, cultura probiótica *L. casei* LC 03 e o extrato de cebola roxa foi pulverizada em $CaCl_2$ 0,1 M por um bico atomizador de fluido duplo (0,1 mm), a 12 cm da solução de $CaCl_2$ sob pressão de ar de 0,125 kgf / cm. Em seguida, as micropartículas foram mantidas sob agitação por 30 min na solução de $CaCl_2$ e peneiradas (0,038 mm (malha 400)).

4.8 CARACTERIZAÇÃO DAS MICROPARTÍCULAS

4.8.1 Microscopia óptica e tamanho médio das micropartículas

A morfologia das partículas foi avaliada utilizando um microscópio óptico (Carl Zeiss Axio Scope.A1, Oberkochen, Alemanha) equipado com uma câmara digital Axio Cam MRc (Carl Zeiss). Já a distribuição do tamanho de partícula foi determinada usando o equipamento de difração a laser Mastersizer 2000 (Malvern, Reino Unido).

4.8.2 Rompimento da partícula e eficiência de encapsulação de polifenois e flavonoides

O rompimento das partículas foi realizado conforme ROBERT et al. (2010) com adaptações. Para isso, uma solução contendo 0.5 mL de acetonitrila e 0.5 mL de metanol: acético: água (50: 8: 42 mL / mL / mL) foi adicionada a 1.0 g de micropartículas. Esta mistura foi agitada em vortex por 4 min e sonicada durante 60 min. Após, o sobrenadante foi coletado e centrifugado a 5000 rpm por 15 min. A partir deste líquido, a eficiência do encapsulação de compostos redutores, flavonoides e antocianinas monoméricas totais foi realizada conforme metodologia descrita nos itens 4.3, 4.4 e 4.5 respectivamente, calculando-se a percentagem de compostos redutores, flavonoides e antocianinas monoméricas totais encapsulados sobre o total destes compostos nas soluções iniciais de alginato.

4.8.3 Eficiência de encapsulação dos probióticos

A eficiência de encapsulação (EE%) dos probióticos será calculada conforme proposto por MARTIN et al., (2013) a partir da Eq. (1).

Eq. 1. $EE = (N/ N_0) X 100$

N= número de células viáveis (log UFC g^{-1}) que foram libertadas pelo rompimento das micropartículas e $N_{0=}$ número de células viáveis (log UFC g^{-1}) no concentrado de células antes da microencapsulação.

4.8.4 Contagem das células viáveis

A liberação das bactérias a partir das micropartículas foi realizada de acordo com o método de SHEU; MARSHALL; HEYMANN, (1993) com modificações. 1 g de amostra adicionado de 9 mL de solução tampão fosfato estéril (pH 7.5) foi homogeneizado por 10 min. Posteriormente, diluições decimais seriadas foram realizadas em água peptonada 0.1% com plaqueamento de 1.0 mL das diluições em triplicata para placas de Petri estéreis, adicionadas de MRS Agar. As placas foram então incubadas a 37 ± 1 °C, por 72 h sob condições de anaerobiose.

4.9 SOBREVIVÊNCIA DOS PROBIÓTICOS EM CONDIÇÕES GASTROINTESTINAIS SIMULADAS

Para avaliar a resistência das partículas frente a condições gastrointestinais simuladas, o método proposto por MADUREIRA et al., (2011) com adaptações foi considerado. 1g de micropartículas ou de polpa de morango foram submetidos a condições simuladas do esôfago / estômago (pH 2.0 / 90 min) na presença de pepsina, condições simuladas do duodeno (pH 5.0 / 20 min) na presença de pancreatina e sais biliares e, condições simuladas do íleo (pH 7.5 / 90 min). Todas as etapas foram realizadas em shaker TE 421 (Tecnal, Piracicaba, SP, Brasil), a 37 ± 2 °C. Para a verificação das contagens probióticas, foram coletadas alíquotas ao final de cada etapa da simulação gastrointestinal 90 min (esôfago / estômago), 110 min (duodeno) e 200 min (íleo) e realizado o plaqueamento de acordo com o item 4.8.4.

4.10 RESISTÊNCIA DAS MICROPARTÍCULAS AO TRATAMENTO TÉRMICO

Para avaliar a resistência térmica dos probióticos, micropartículas e cultura livre (1 g) foram transferidos para 9 ml de água peptonada estéril em tubos de ensaio. O conteúdo foi submetido a condições térmicas de 72 ° C por 15 seg e 63 ° C por 30 min, em seguida, os tubos foram resfriados por imersão em gelo por 10 min. Alíquotas foram coletadas e as culturas probióticas contadas de acordo com a Seção 4.8.4 (ZHANG; LIN; & ZHONG, 2015).

4.11 AVALIAÇÃO DAS MICROPARTÍCULAS EM DIFERENTES CONDIÇÕES DE ARMAZENAMENTO

Para avaliar a estabilidade do probiótico nas micropartíulas, contagens foram realizadas a cada 15 dias de acordo com o item 4.8.4 com as amostras armazenadas em ependorfs estéreis e protegidas da incidência de luz a 25 ± 1 °C, 7 ± 1 °C e -18 ± 1 °C, que simulam condições de temperatura ambiente, de refrigeração e de congelamento respectivamente, por um período de 90 dias. Já a viabilidade dos probióticos na polpa de morango foi avaliada pelo período de 60 dias a -18 ± 1 °C, com contagens realizadas a cada 15 dias.

4.12 ELABORAÇÃO DE POLPA DE MORANGO COM ADIÇÃO DE MICROPARTÍCULAS PROBIÓTICAS

Para a elaboração da polpa, morangos orgânicos foram adquiridos na cidade de Agudo (RS) e higienizados com água corrente, processados em liquidificador e peneirados. Em seguida, 5% de micropartículas foi adicionado à polpa e homogeneizado (5 g / 100 g). A

polpa foi então acondicionada em sacos de polietileno e armazenada a –18 °C para posteriores análises.

4.13 ANÁLISES MICROBIOLÓGICAS DA POLPA DE MORANGO

As detecção de *Salmonella* sp., coliformes totais, coliformes termotolerantes e bolores e leveduras, foram realizadas de acordo com BRASIL, (2001). Já a quantificação dos probióticos foi realizada de acordo com SHEU, MARSHALL, & HEYMANN (1993) com modificações. Os probióticos foram libertados das micropartículas com solução tampão de fosfato estéril (pH 7,5), posteriormente, diluições decimais em série foram realizadas em água peptonada a 0,1% com plaqueamento de 1,0 mL das diluições em triplicata, em seguida adicionadas com Ágar MRS e incubadas a 37 ± 1 °C por 72 h em condições anaeróbias.

4.14 ANÁLISES FÍSICO-QUÍMICAS DA POLPA DE MORANGO

Foi utilizada a metodologia do Instituto Adolfo Lutz (IAL, 2009) para avaliação do pH, sólidos solúveis, umidade e acidez titulável da polpa.

4.15 ANÁLISE ESTATÍSTICA

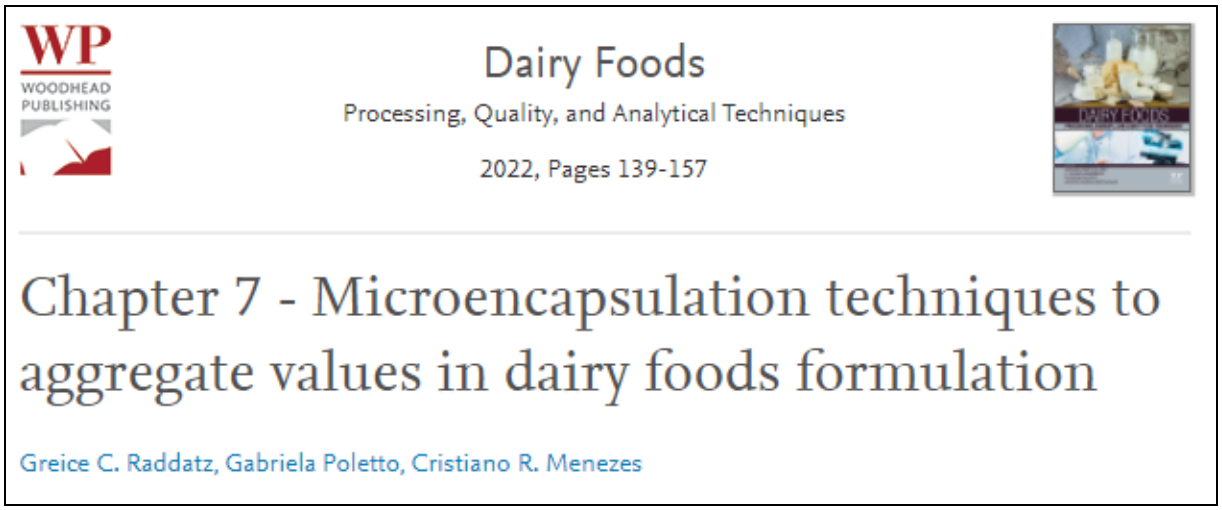
Os dados serão submetidos à análise de variância (ANOVA), no software Statistica versão 7.0 (2004; Statsoft Inc., Tulsa, OK, EUA), seguido pelo teste de comparação de médias de Tukey, considerando o nível de significância de 5% ($p < 0.05$). Todos os testes serão realizados em triplicata e os dados expressos em média ± desvio padrão.

5. CAPÍTULOS DE LIVRO E ARTIGOS CIENTÍFICOS INTEGRADOS

5.1 CAPÍTULO I –

MICROENCAPSULATION TECHNIQUES TO AGGREGATE VALUES IN DAIRY FOODS FORMULATION

Capítulo publicado no livro Dairy Foods: Processing, Quality, and Analytical Techniques.



Microencapsulation techniques to aggregate values in dairy foods formulation

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Abstract

Nowadays, consumers are looking for varied, nutritious, healthy and as natural foods as possible. This behavioral change requires the food industry to adapt to the profile of this new consumer, and should invest in the preparation of foods with potential health benefits. Dairy products, in addition to being recognized for their functional character, are well accepted and consumed almost everywhere in the world, thus, the production of dairy products containing bioactive compounds raises the productivity margin and creates new opportunities for this sector. Microencapsulation meets consumer expectations, since it allows the insertion of the most diverse active components (probiotics, prebiotics, antioxidant compounds, vitamins, essential oils, among others) in food matrices, contributing to the development of products with added value and that promote well-being and health.

Key Words: bioactive, microcapsules, functional foods, dairy products, viability.

1. Introduction

The search for consumers for a healthy diet, which favors physical well-being and helps in the prevention of diseases, causes the food industry to seek more and more innovations, a fact that has caused an increase in the functional food market, as these , in addition to their basic nutritional functions, confer consumer health benefits (Abd-El-Salam; El Shibiny, 2015).

The substance that has a functional property can be naturally present in the food or be added / modified, so that it can perform one or more specific functions in the body. Thus, functional foods can be conventional foods added with bioactive ingredients capable of generating health benefits and reducing the risk of developing chronic diseases (Alvim; Fadini; Cruz, 2014).

Recent research has shown that milk and its derivatives (cheeses, yogurts, butter, etc.) have numerous bioactive components potentially beneficial to human health that go beyond the supply of nutrients and energy required to maintain basal metabolism and growth. Dairy products are considered the most basic products in the human food chain. According to FAO (2018), milk production in the world increased by 0.5% in 2017 and is expected to grow by 22% by 2027. In view of the economic, nutritional and technological importance of milk and its derivatives, the application of bioactive compounds can be an alternative means for the development of new functional foods.

Bioactive compounds (probiotics, essential oils, vitamins, minerals, polyphenols, carotenoids, etc.) are substances obtained from fruits, vegetables, oils, microorganisms and other sources and have beneficial effects on health through cellular and physiological activities (Wei & Huang , 2019). They have antioxidant, anti-inflammatory, antibacterial and immunomodulatory activities (Li, Bao, & Chen, 2018), preventing metabolic, coronary diseases, urinary tract infections, stomach ulcers, dental diseases, as well as various forms of cancer (Shishir, Xie, Sun, Zheng & Chen, 2018).

However, the incorporation of bioactive compounds in food formulations faces some challenges, mainly with regard to the stability of these compounds during the processing and storage of food products (Champagne; Fustier, 2007). Many of these compounds are easily degraded when exposed to light, heat and pH, which limits their application. An alternative that has been shown to be efficient to protect bioactive compounds from these adverse conditions, maintaining their viability and functionality, is microencapsulation (Aguiar; Estevinho; Santos, 2016; Ballesteros et al., 2017).

Microencapsulation is based on the coating or packaging of a solid, liquid or gaseous material within another material, which can be used in the food industry for several reasons such as: protection of the trapped active agent against loss of nutritional value and interaction with harmful environmental factors (for example, heat, light, air and humidity); decreased evaporation and transfer rate of the main agent to the outside; physical properties of original substances can be modified, such as converting liquids into a dry solid system, for easy handling; the release of the core material can be controlled as a delayed (time) or long-acting (sustained) release; mask unpleasant organoleptic properties, such as the odor and taste of some compounds; prevention of incompatibility between components of the trapped mixture; the encapsulated agent can be diluted when a very small amount is needed (Narsaiah et al., 2014).

Therefore, the objective of this chapter is to demonstrate different encapsulation techniques for the protection and delivery of bioactive compounds intended for application in dairy products.

2. Dairy Market

Milk has been part of the daily diet of consumers since its birth and its consumption and its derivatives is strongly recommended since it represents benefits for the health of the population of different age groups. Dairy products are a source of great nutritional value, which contribute to a diet rich in nutrients, acting as components of a healthy diet (Barlowska et al., 2011).

Milk and milk products offer quality protein, minerals, vitamins and energy (Niro et al., 2017; Slacanac et al., 2011). In addition, about 70% of daily calcium replacement is obtained by consuming these products (Huth & Rienzo 2006), which is an indispensable compound for the formation and maintenance of bones, in addition to other functions.

However, consumers increasingly prioritize the daily intake of foods that offer different health benefits, in addition to their high nutritional value. Faced with this new scenario, as a way of adapting to the trends of the consumer market, in addition to increasing competitiveness and maintaining technological leadership, the dairy industries seek to develop new formulations with a functional character, capable of satisfying this demanding market (Thamer & Penna, 2006; Oliveira et al., 2018).

Dairy products are well known and accepted among the world population, thus, the development of innovative, healthy and functional products in this sector, would further contribute to the consumption of milk and dairy products.

3. Microencapsulation

Microencapsulation consists of the formation of solid particles with small diameters, containing in their interior, one or more active ingredients (Łozińska, Głowacz-Różyńska, Artichowicz, Lu, & Jungnickel, 2020). Thus, in a microcapsule, the active material is known as the nucleus and the material that surrounds it is called wall (Dubey, Shami, & Bhasker Rao, 2009).

Microcapsules are usually more resistant to certain environmental factors in which the active compound may be exposed during its production, storage and consumption, such as, for example, extreme temperatures, acidic pH and oxygen, allowing extended storage and longer

compound shelf life (Feyzioglu & Tornuk, 2016) in addition, microcapsules allow the release and delivery of the active compound only to its site of action (de Menezes et al., 2013).

The release of the active agent from inside the microcapsules can occur through different triggers, which lead to the rupture or total dissolution of the capsules, of which the pH control, use of temperature, pressure and also through diffusion and degradation stand out (Desai & Park, 2005).

For the wall material selection, some factors must be considered, the compost must be food grade, must guarantee the release of the nucleus in its active site, should not react with the core material and must be selected taking into account the desired encapsulation technique and the ability to protect the encapsulated material from unfavorable conditions (Sánchez, García, Calvo, Bernalte, & González-Gómez, 2016).

The encapsulating materials can be of natural, synthetic or semi-synthetic origin, in which carbohydrates, lipids, proteins and synthetic polymers stand out, as shown in Table 1.

Table 1. Wall materials used in microencapsulation (Suave et al., 2006; Martins, Barreiro, Coelho, & Rodrigues, 2014).

Carbohydrates	**Proteins**	**Lipids**	**Synthetic polymers**
*Starches *Dextrins *Corn Syrup * Celluloses *Gums (arabic, alginate, carrageenan)	*Gluten *Casein *Gelatine *Albumin	*Wax *Paraffin *Tristearin * Stearic acid *Monoglycerides/diglycerides *Oils *Hydrogenated fats	*Poli D *L-lactic acid * Polyacrylates *Co-propylene polyethylene copolymers *Poly ε-caprolactone

3.1 Microencapsulation techniques

Different techniques can be used for the production of microcapsules, the selection of the method will depend on a series of factors such as the intended application, the selected wall material, the methodology and the release site of the active compound, the production costs, the physical-chemical nature of the active material, among others (Mahdavi, Jafari,

Ghorbani, & Assadpoor, 2014). In addition, each microencapsulation technique can result in particles with unique characteristics, with differences in their shape (spheres, films, irregular particles), in their structure (porous or compact), their water content, size, among other characteristics , which can influence the stability and delivery of the active compound (Madene, Jacquot, Scher, & Desobry, 2006).

As shown in Figure 1, the classification of the obtained microparticles can be divided based on the distribution of the encapsulated material in the particle, it can be a microcapsule, where the nucleus remains trapped in the center of the particle and is surrounded by wall material, or it could be a microsphere, which is considered a matrix system, and the core material is evenly distributed across the wall material, also being exposed to the outside of the particle (Azeredo, 2005).

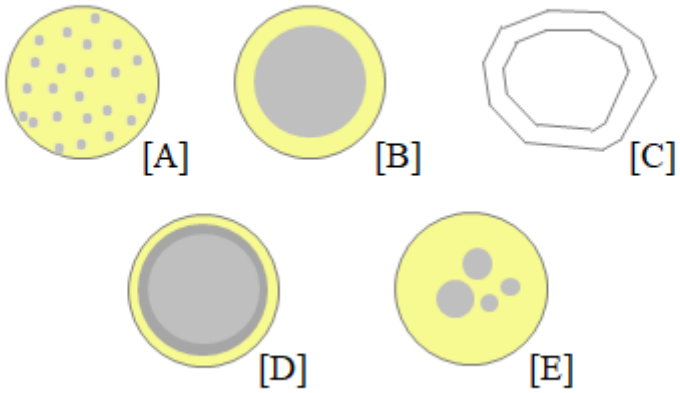

Figure 1 - Microparticle morphology. A = microsphere; B = simple microcapsule; C = irregular simple microcapsule; D = microcapsule with double wall; E = polynucleated microcapsule. Source: Azeredo, (2005).

Different methodologies are described for the microencapsulation of bioactive substances, which are divided into three groups as shown in Table 2.

Table 2. Techniques used for the production of microcapsules (Ré, 1998; Jyothi et al., 2010; Madene, Jacquot, Scher, & Desobry, 2006; Barreto, Ramírez-mérida, Etchepare, Jacob-Lopes, & Menezes, 2015).

Chemical	Physical	Physicochemical

*Interfacial polymerization *Molecular inclusion *In situ polymerization	*Spray drying *Spray chilling *Spray coating *Ionic gelation * Co-crystallization *Freeze-drying *Fluidized bed coating *Centrifugal extrusion	*Coacervation *Emulsion-solvent evaporation *Solidification emulsion *Liposomes

3.1. 1 Ionic gelation

The formation of microcapsules through ionic gelation can occur in two ways, by internal ionic gelation also known as emulsification or by external ionic gelation or extrusion, both consist of the formation of microcapsules in the presence of multivalent ions such as Ca^{2+}, Ba^{2+} and Al^{3+}, which cause the crosslinking of polyelectrolytes present in the encapsulating solution (Yeo, Baek, & Park, 2001; Lupo, Maestro, Gutiérrez, & González, 2015).

In external ionic gelation / extrusion, multivalent ions diffuse with the encapsulating solution from an external source (Davarcı, Turan, Ozcelik, & Poncelet, 2017). As shown in Figure 2, for the production of microcapsules, the material to be encapsulated is homogenized with the encapsulating material and passes through an extruder that disperses this mixture in small droplets. The droplets come into contact with a gelling solution (usually calcium chloride) and are then filtered and separated from the gelling solution.

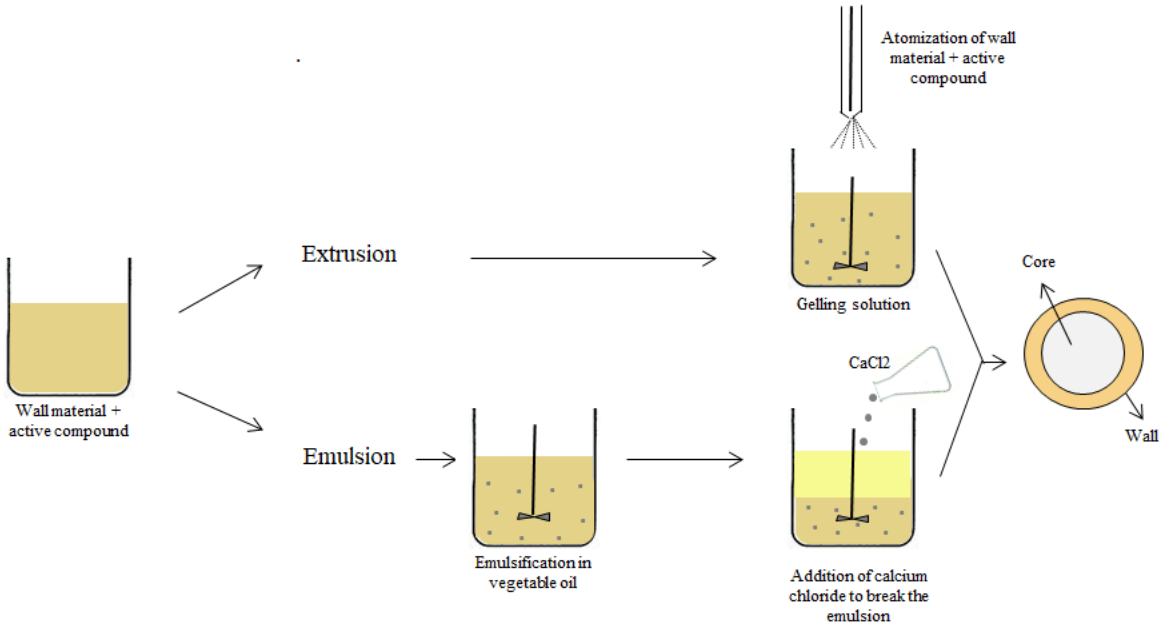

Figure 2 - Stages of the encapsulation process by means of extrusion and emulsification techniques. Adapted from: (Coultata, 1984; Gaserod et al., 1998).

In this technique, natural polysaccharides are commonly used, in addition, the use of organic solvents and extreme temperatures are not necessary for the production of microcapsules, which makes this technique widely explored in food area (de Araújo Etchepare et al., 2020).

The internal ion gelation / emulsification process, on the other hand, produces particles based on the formation of an emulsion of two or more immiscible liquids. The wall material together with the active compound are introduced into a vegetable oil under agitation, forming a water-in-oil emulsion. In this step, adding a calcium salt, the crosslinking of the wall material occurs and the microcapsules are formed. As the calcium salt is added dropwise to the polymeric solution, the capsules produced will have an aqueous core. The formed microcapsules must then be separated from the oily phase and washed for total oil removal (Rokka & Rantamäki, 2010).

The main polymers used for the production of microcapsules by ionic gelation are alginate, pectin and carrageenan (Doleyres & Lacroix, 2005).

3.1.2 Spray drying and Freeze-drying

The Spray Drying and Frezze-drying processes allow the production of dry microcapsules by evaporating the solvents present in the encapsulating solution. Both techniques are capable of reducing the product's water activity, resulting in a prolonged shelf life, high microbiological stability, and consequently reduced component degradation, as well as reducing storage and transportation costs (Aliakbarian, Paini, Casazza, & Perego, 2015). Despite this, these two processes can result in products with totally different characteristics, since these techniques operate under different temperature and time conditions (Kuck & Noreña, 2016).

Spray drying is done by accessible equipment (spraydryer), which allows a relatively low production cost, being a fast and efficient technique for the formation of particles (Dziezak, 1988; Campelo, Sanches, Fernandes, Botrel, & Borges, 2018). In this technique, the encapsulating solution together with the active compound are atomized under high temperatures and lose their water content in a few seconds, being transformed into dry microparticles. Allowing the production of dry and agglomerated foods, this method is widely used in the food industry (Shishir & Chen, 2017).

The process for obtaining microcapsules from spray drying occurs in three fundamental steps; first, the formation of droplets occurs, in a second moment, the droplets come into contact with high temperatures, which lead to the evaporation of the solvent, finally, the dry particles are formed and separated (Stunda-Zujeva, Irbe, & Berzina-Cimdina, 2017). As shown in Figure 3, the encapsulating solution together with the active material are atomized to a drying chamber with constant circulation of hot air, the particles then come into contact with this air. In this step, the hot air increases the temperature of the droplets and results in the evaporation of the solvent contained in the solution, the solution is transformed into solid particles that surround the active material forming the microcapsules. These particles are then separated from the exhaust air by a cyclone and collected (Shishir & Chen, 2017).

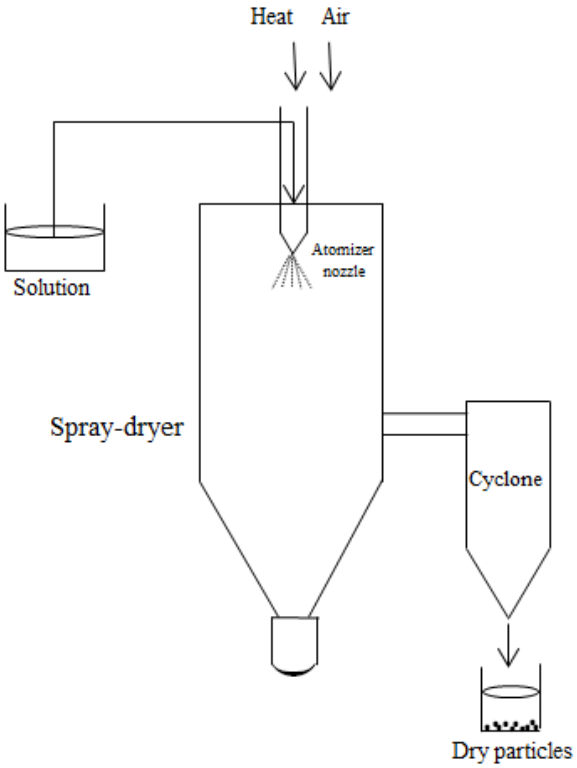

Figure 3 - representation of the microencapsulation process by spray drying. Source: Adapted from (Estevinho et al., 2013).

On the other hand, the Frezze-drying process consists of removing water or other solvent from the product through the sublimation process (Massounga Bora, Ma, Li, & Liu, 2018). It is known to be a simple technique for dehydrating heat-sensitive compounds and materials and can also be used for the production of microcapsules (Desai & Park, 2005). In this case, the encapsulating material together with the active compound is deposited in the freeze dryer, where the removal of the frozen solvent is done through sublimation and removal of the water molecules linked by desorption, under conditions of low temperature and pressure. In this process, with the loss of water from the material, formation of microparticles occurs, involving the active compound.

Frezze-drying is commonly used in the food industry, being less harmful to substances that are not stable at high temperatures or in the presence of water, in addition, the dry product obtained has low apparent density and good retention of some compounds (Horszwald, Julien, & Andlauer, 2013). In the case of developing microcapsules containing compounds sensitive to freezing, as is the case of probiotic cells, for example, the use of cryoprotectants is advisable to reduce damage to the active compound (Manojlović, Nedović, Kailasapathy, &

Zuidam, 2010). Despite the advantages like be a simple technique, do not use heat and air, which reduces the oxidation of compounds, the cost of this methodology is increased, as well as the time required for product formation (more than 20 h) (Lopez-Quiroga, Antelo, & Alonso, 2012).

3.1.3 Complex coacervation

Complex coacervation is an encapsulation technique based on the interaction, in an aqueous medium, between two oppositely charged polyelectrolytes, where the complex between these materials (normally proteins and polysaccharides) is formed around droplets or particles of the active material to be encapsulated under certain specific conditions such as pH, ionic strength, concentration of polymers, ratio of biopolymers, molecular weight of biopolymers, temperature and degree of homogenization, as described in the Figure 4. (Marques da Silva et al., 2018; Timilsena et al., 2016). This technique allows to obtain reservoir-type particles, where the nucleus is surrounded by a layer of encapsulating materials (Fávaro-Trindade; Pinho; Rocha, 2008).

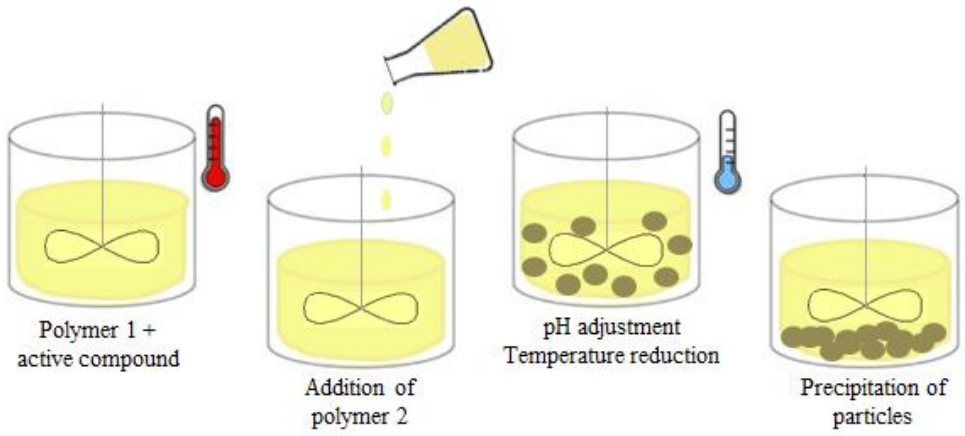

Figure 4 – Schematic representation of the microencapsulation process by complex coacervation. Adapted from: (Araújo, 2011)

The proteins often used to produce complex coacervates are gelatin, albumin, whey protein, beta lactoglobulin and various plant proteins. Common polysaccharides are gum arabic, chitosan, pectin, alginates, xanthan gum, carrageenan and carboxymethyl cellulose (Kaushik et al., 2016).

It is widely used for encapsulating aromas and hydrophobic compounds, however it has the potential to encapsulate other types of compounds, such as water-soluble vitamins,

sweeteners and phenolic compounds (Alvim; Grosso, 2010; Nori et al. 2011; Comunian et al. 2013; Rocha-selmi et al. 2013).

The method has many advantages when compared to the others, such as versatility, high encapsulation efficiency, efficient control of particle size, possibility of working with biopolymers, absence of organic solvents, low cost and simplicity (Shoji et al., 2013). In addition, the microcapsules produced by coacervation have controlled release characteristics, modulated by changes in ionic strength, pH and temperature (Bosnea, Moschakis, Nigam & Biliaderis, 2017; de Menezes et al., 2013).

3.1.4 Liposomes

Liposomes are spherical structures capable of encapsulating hydrophilic and hydrophobic substances, since they consist of an external phase containing a system of phospholipid membranes and an internal phase consisting of an aqueous medium (Bejarano & Diaz, 2004).

These structures are obtained from amphiphilic substances, capable of forming a lamellar phase (Machado, Gnoatto, & Klüppel, 2007) and its preparation can occur through REV (vesicles obtained by evaporation in reverse phase), FPV (vesicles obtained in a French press) and EIV (vesicles obtained by ether injection) (Lasic, 1998; Vemuri & Rhodes, 1995).

As an advantage of this methodology, we can mention the delivery of products (lipids and phospholipids) with guaranteed quality, large-scale production capacity, high stability of liposomal preparations, as well as allowing the development of sterile liposomal products (Wagner, Vorauer-Uhl, & Katinger, 2002).

4. Incorporation of microencapsulated bioactive compounds in dairy products

4.1. Probiotics, prebiotics and symbiotics

Research demonstrating the effects of intestinal microflora on human well-being and health has turned the population's attention to the search for functional microorganisms known as probiotics (Cheng, Song, Xie, & Song, 2019). According to the definition by (Hill et al. (2014) probiotics are “live microorganisms that, when administered in adequate amounts, confer positive effects on the host”, with an improvement in the immune response (Bogsan et al., 2014), balance in the intestinal microbiota composition (Omar, Chan, Jones, Prakash, & Jones, 2013), serum cholesterol reduction (Agrawal, 2005), reduced inflammation

(Fioramonti, Theodorou, & Bueno, 2003; Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013), improve lactose intolerance (Kechagia et al. 2013), among many other benefits.

The minimum amount of 10^6-10^7 CFU / g or ml of viable cells at consumption time is required for the product receive the definition of probiotic (Bertazzoni, Donelli, Midtvedt, Nicoli, & Sanz, 2013). This dose can be achieved through microencapsulation, which guarantees the delivery of these microorganisms to their action place in required quantities, in addition to protecting them from external factors and increasing their shelf life, since probiotic-free cells are highly susceptible to disintegration when added to food formulations (Bampi et al., 2016). Among the existing probiotics, those belonging to the genera *Lactobacillus* and *Bifidobacterium* are the main known and used microorganisms (Trabelsi et al., 2013).

A product that combines probiotic microorganisms and prebiotic compounds, meets the definition of symbiotic. Prebiotics are compounds fermentable by microorganisms present in the colon (probiotics), exerting synergistic effects and stimulating their growth (Bersaneti, Garcia, Mali, & Pedrine Colabone Celligoi, 2019). In addition, studies have already reported that prebiotic components are able to increase the viability and stability of microcapsules containing probiotics (Poletto et al., 2019).

Ribeiro et al. (2014) added *Lactobacillus acidophilus* LA-5 microencapsulated by ionic gelation and complex coacervation using pectin and whey protein, in stirred probiotic yogurt and evaluated the product's physical-chemical, sensory and microbiological characteristics. The authors reported that the encapsulated probiotic had less post-acidification, longer survival after 35 days of cold storage, as well as longer survival during simulated gastrointestinal conditions, when compared to the free probiotic, in addition, the product added from the microcapsules had a good overall impression.

Dimitrellou et al. (2019) developed a fermented milk containing *Lactobacillus casei* ATCC 393 in alginate microcapsules obtained by extrusion and evaluated the survival of the probiotic to simulated conditions of the gastrointestinal tract and its stability in the product for four weeks at 4 ° C. High probiotic survival was observed after product processing and storage, greater viability against simulated gastrointestinal conditions compared to free cells, and the sensory characteristics of the product were not affected by the addition of microcapsules.

In addition, other dairy products added with probiotic microcapsules have been studied, such as, for example, buffalo milk yogurt containing *L. acidophilus* by complex coacervation followed by freeze-drying (Shoji et al., 2013), ice cream with *Lactobacillus*

rhamnosus R0011 and *Bifidobacterium longum* R0175 (Champagne, Raymond, Guertin, & Bélanger, 2015), Frozen iogurt containing *Bifidobacterium* BB-12 by spray drying (Pinto et al., 2012), in yellow mombin ice cream containing *Lactobacillus rhamnosus* and *Lactobacillus casei* by extrusion (Farias et al., 2019), among others.

Likewise, dairy products containing symbiotic microcapsules have aroused researchers' interest in the development of new functional foods, for example, Mirzaei, Pourjafar, & Homayouni, (2012) developed a Iranian white brined cheese containing free and microencapsulated *Lactobacillus acidophilus* La5 by extrusion with calcium alginate and resistant starch (Hi-maize), and reported that microencapsulation allowed less reduction of viability in the cheese during the storage of 6 months.

Meanwhile, Fritzen-Freire et al. (2013) evaluated the addition of microcapsules containing *Bifidobacterium* BB-12 by spray drying with reconstituted skim milk and the prebiotics inulin and inulin enriched with oligofructose in ricotta cream. The encapsulated probiotic showed much higher cell viability than the free one during the storage period (60 days), in addition, the ricotta cream showed good acceptability, as a result of the sensory analysis performed.

Another product evaluated by Homayouni, Azizi, Ehsani, Yarmand, & Razavi, (2008) was symbiotic ice cream containing *L. casei* and *B. lactis* by internal gelation with alginate and the Hi-maize prebiotic stored for 180 days at 20 ºC, they concluded that encapsulation increased the probiotics' viability period.

Therefore, these studies demonstrate that the use of means of protection becomes indispensable for the safe delivery and the guaranteed viability of probiotic microorganisms, with microencapsulation being an effective means for this, in addition to enabling the development of new bioactive foods for the consuming public.

4.2. Antioxidants

Free radicals in the human body can cause neurodegenerative, cardiovascular, mitochondrial diseases and even cancer (Sanjukta & Rai, 2016), thus, there was growing interest in attempts to understand the mechanism of action of free radicals and to find effective substances that protect organisms against their harmful influence (Shalaby & Shanab, 2013).

Antioxidant compounds are substances that can interfere in oxidative cycles to inhibit or delay oxidative damage in biomolecules, and can prevent before the formation of reactive species, eliminate free radicals, form chelate complexes with pro-oxidant metals, extinguish

singlet oxygen and photosensitizers, deactivate or activate enzymes, remove and repair damage caused by reactive species (Polumbryk et al., 2013).

The main classes of natural compounds with antioxidant activity are: vitamins (C and E), carotenoids (carotenes and xanthophylls) and polyphenols (flavonoids, phenolic acids, lignans and stilbenes) (Sindhi et al., 2013). However, several limitations need to be overcome in order to increase the use of these compounds in products on the market, such as instability problems such as autooxidation reactions, epimerization, instability to pH variation and gastric content, in addition to low bioavailability (Chanchal; Swarnlata, 2009; Wisuitiprot et al., 2011). Thus, microencapsulation enters as an ally for its application in products, mainly in dairy products since they are widely consumed worldwide due to its composition rich in proteins, minerals and vitamins (Niro et al., 2017).

Feng, Li, Wang, & Zhu, (2018) microencapsulated *Phaffia rhodozyma* astaxanthin with sodium carboxymethylcellulose (CMC-Na) and microcrystalline cellulose (MCC) by lyophilization and added in yogurt. The authors observed that encapsulation improved the stability, solubility and antioxidant activity of astaxanthin and increased the extent of its potential industrial application. This study corroborates that of Taksima et. al. (2015) who added natural astaxanthin from shrimp residues microencapsulated by ultrasonic atomizer using alginate-chitosan, in yogurt and evaluated the sensory profile, acceptability and purchase intention of the consumer. The authors reported that the encapsulation significantly increased the stability of natural astaxanthin with potential use as a functional ingredient in yogurt, since there was good acceptance by consumers.

Çam, İçyer, Erdoğan (2014) microencapsulated pomegranate peel extract using the *spray driyng* technique and maltodextrin as the wall material and incorporated the microparticles in ice cream. The authors reported that during the 90 days of storage, ice cream added with microparticles containing 0.5 and 1.0% (w / w) of phenolic extracts of pomegranate showed better antioxidant and a-glucosidase inhibitory activity when compared to the control sample. As for the sensory evaluation, more than 75% of the participants accepted ice cream containing microparticles, which makes this product a potential for the development of new functional foods.

4.3. Enzymes

Lactase (β-Galactosidase) is an enzyme produced in the small intestine, responsible for the hydrolysis of lactose (5% w / w in milk) in glucose and galactose (Scheppingen-Van et al., 2017). Due to the lack of β-galactosidase, more than 70% of the world population cannot

easily digest milk, (Adilac, 2016). The natural loss of lactase activity with age (primary lactase deficiency) or due to active gastrointestinal diseases, medications or intestinal surgeries (secondary lactase deficiency) leads to lactose malabsorption. Undigested lactose enters the colon, where it is fermented by colon bacteria in gases (hydrogen, methane and carbon dioxide) and short-chain fatty acids. Excessive gas production can cause abdominal pain, cramps, swelling or vomiting, while high osmotic load in the colon causes diarrhea, this problem is known as lactose intolerance (Pawłowska, Umławska, & Iwańczak, 2016).

To get around this problem, the content of lactose in milk and dairy products is usually reduced by enzymatic or chemical hydrolysis. Enzymatic hydrolysis of lactose does not impair the nutritional value of dairy products and produces less unpleasant flavors, odors and colors than chemical hydrolysis. However, the direct addition of free or immobilized β-galactosidase to milk results in the action of hydrolysis to generate the constituent monosaccharides, glucose and galactose, which makes the milk very sweet. In order to maintain the characteristic flavor of whole milk and protect the enzymatic activity of proteolysis in the stomach, the microencapsulation of β-galactosidase has been investigated by many researchers (Ahn, Lee & Kwak, 2019; Rodriguez-Nogales; Delgadillo, 2005).

Ahn, Lee & Kwak (2013) microencapsulated lactase (β-galactosidase) by multiple emulsions (W / O / W), in order to avoid lactose intolerance in milk. The core material was lactase and the coating materials were medium chain triglycerides for the water / oil phase, and whey protein isolate, maltodextrin, gum arabic and their mixtures for the A / O / A phase. Polyglycerol polyricinoleate (PGPR) was used as a primary emulsifier and polyoxyethylene sorbitan monolaurate (PSML) was selected as a secondary emulsifier based on the emulsion stability index. Based on the data obtained in this study, the lactase microcapsules showed effective results, which can be a method to produce new products for people who have lactose intolerance.

In another study, Zhang et al., (2016) microencapsulated b-galactosidase by injecting an aqueous solution, containing b-galactosidase and carrageenan (1% by weight), in a 5% solution of potassium chloride. About 63% of b galactosidase was encapsulated in the hydrogel beads. The encapsulated b-galactosidase had a higher activity than the free enzyme in a range of pH and thermal conditions, which was attributed to the stabilization of the enzyme structure by the K + ions in the carrageenan spheres. However, the release of the enzyme from the spheres was observed during storage in aqueous solutions, which was attributed to the relatively large pore size of the hydrogel matrix. Thus, the results suggest that

the carrageenan hydrogel spheres may be useful encapsulation systems, but further studies are needed to inhibit enzyme leakage.

4.4. Other applications

In addition to the demand for dairy products with a digestive functionality appeal (with the addition of prebiotics, probiotics and symbiotics), several studies have also demonstrated the demand for dairy products for other functions, such as the addition of antioxidant compounds and enzymes, mentioned in this chapter. In addition, there is an enormous potential for the use of other functional components for the development of foods, beverages and dietary supplements such as sulfur and nitrogen compounds, phenolic compounds, polyunsaturated fatty acids (highlighting the omega-3 and 6 series), fibers, proteins, peptides and amino acids which will be presented in studies to follow.

Rutz et al. (2017) microencapsulated palm oil, containing a high content of carotenoids, using the complex coacervation method and chitosan / xanthan gum and chitosan / pectin as wall materials. Subsequently, the efficiency of the encapsulation and the internalization of the carotenoids, the morphology, the thermal behavior and the release profile of the carotenoids in water and in a fluid that simulates the gastrointestinal environment were evaluated. In addition, microparticles were applied in the preparation of bread and yogurt and the release profile of encapsulated compounds was evaluated when fortified foods were exposed to fluids that simulate gastrointestinal conditions. The authors reported that chitosan / xanthan gum microparticles had the best potential to be successfully applied in the food industry, particularly in yogurt preparations.

Comunian et. al. (2017) coencapsulated echium oil, phytosterols and synapic acid (crosslinker / antioxidant) by complex coacervation and incorporated the microcapsules obtained in the yogurt. Microcapsules were evaluated for particle size, oxidation accelerated by Rancimat and simulation of gastric / intestinal release. Yogurts were evaluated for morphology, pH, titratable acidity, color, rheology and sensory analysis. It was observed that the physical-chemical, rheological and sensory properties of the yogurt with added microcapsules were similar to the yogurt control (without the addition of bioactive compounds) and superior to the yogurt with the non-encapsulated bioactive added.

Da Silva et al. (2019) microencapsulated *Spirulina platensis (*microalgae) by spray using two encapsulation materials: maltodextrin and maltodextrin cross-linked with citric acid. Subsequently, free and encapsulated *Spirulina platensis* were evaluated for their bioactive properties and added to yogurts. The authors reported that the incorporation of *S.*

platensis in its encapsulated form was more efficient than its direct use in yogurt. In addition to the greater homogeneity of the yogurts, it was possible to conclude that microencapsulation maintained the nutritional profile and improved the antioxidant activity over the storage time.

In a study by Gupta et al. (2015), iron microcapsules were prepared using a mixture of gum arabic, maltodextrin and modified starch by the modified solvent evaporation method. These microcapsules were added to the milk and evaluated for sensory characteristics and oxidative stability. It was observed that milk enriched with microcapsules showed significantly higher bioavailability of iron in vitro compared to control milk (not fortified) and was also better accepted sensorially.

Using corn zein protein (Zea mays) as wall material, Xiao, Davidson and Zhong (2011) microencapsulated nisin and thymol (antimicrobials) by *spray drying*. The microcapsules were added in low fat milk at 25 °C and tested for their antimicrobial activity. The authors reported that the microcapsules exhibited antimicrobial activity and inhibited the growth of *Listeria monocytogenes*.

In addition, it is well known that a large part of the population faces an increasingly fast pace of life, which makes it necessary for their daily lives to have practical and easily accessible foods, such as Snacks, which include snacks with / with dairy products. , and at the same time to be healthy, driving the need for products with low sodium / sugar / fats. Microencapsulation also comes as a platform in the development of these products.

4. Conclusion

Microencapsulation has been a promising technology in the incorporation of numerous active ingredients in dairy products. This methodology allows extending the viability of probiotic microorganisms, protecting them until they reach the ileum, increasing bioavailability as well as incorporating antioxidant compounds in foods, in order to enrich the composition of this matrix and promote health, in addition, it allows incorporating enzymes in food, enabling its consumption to the public intolerant to certain compounds. Microencapsulation protects these compounds against adverse conditions such as temperature, oxygen, passage through the gastrointestinal tract, without changing the texture and sensory characteristics of the product, enabling the development of products with high acceptability. Many active ingredients do not yet have a tested application in dairy products, so research on this is necessary to further expand this very promising market and to allow the provision of new foods with the most diverse compounds for the consumer.

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5.2 ARTIGO I –

MICROENCAPSULATION AND CO-ENCAPSULATION OF BIOACTIVE COMPOUNDS FOR APPLICATION IN FOOD: CHALLENGES AND PERSPECTIVES

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Microencapsulation and co-encapsulation of bioactive compounds for application in food: challenges and perspectives

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Microencapsulation and co-encapsulation of bioactive compounds for application in food: challenges and perspectives

Microencapsulação e co-encapsulação de compostos bioativos para aplicação em alimentos: desafios e perspectivas

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ABSTRACT

The availability of different food products containing bioactive compounds promotes their inclusion in the daily diet of consumers. However, the effective and safe delivery of such products requires certain precautions to ensure their preservation, stability, and bioavailability when consumed. Microencapsulation is a great alternative, which is a method capable of protecting different bioactive compounds, including probiotic cells, prebiotic compounds, and some antioxidant substances such as phenolic compounds, anthocyanins, flavonoids, and vitamins. Therefore, this study aimed to perform a literature review and present different alternatives to make bioactive compounds viable through microencapsulation, increase their stability and viability when applied in different food matrices, and address the existing challenges regarding their effectiveness.

Keywords: microcapsules, probiotics, prebiotics, antioxidants, co-encapsulation.

RESUMO

A oferta de diferentes produtos alimentícios que contenham compostos bioativos facilita a sua inserção na dieta como parte do dia a dia do consumidor, no entanto, para que estes compostos sejam entregues de forma segura e eficaz, o uso de certos meios se torna necessário para garantir sua preservação, estabilidade e biodisponibilidade quando consumidos. Com esta finalidade, apresenta-se como uma grande alterativa a microencapsulação, que é um método capaz de fornecer proteção a diferentes compostos bioativos, que incluem células probióticas, compostos prebióticos, e algumas substâncias antioxidantes como compostos fenólicos, antocianinas, flavonoides, vitaminas, dentre outros e garantir uma melhor efetividade na sua entrega. O objetivo deste trabalho é realizar uma revisão apresentando formas de viabilizar os compostos bioativos através da microencapsulação, para aumentar sua estabilidade e viabilidade diante da aplicação em diferentes matrizes alimentícias, além de abordar os desafios existentes para a sua efetividade.

Palavras-chave: microcápsulas, probióticos, prebióticos, antioxidantes, co-encapsulação.

INTRODUCTION

Bioactive compounds are important substances due to their role in disease prevention and functional profile (BAO et al., 2019). In this context, nutritional and functional foods have become a promising niche in the current market due to new trends in consumer eating habits. Within the group of functional compounds, which is quite broad, probiotic bacteria, prebiotic compounds, and antioxidant compounds deserve to be highlighted.

Probiotics are live microorganisms that provide positive effects to the host when ingested in adequate amounts (HILL et al., 2014). Prebiotics, on the other hand, are non-digestible food components that selectively stimulate probiotic growth (ROBERFROID et al., 2010). Symbiotics are a combination of probiotics and prebiotics that confer beneficial effects to the immune system against pathogenic microorganisms, in addition to their role against some types of cancer and anti-allergic action (OLIVEIRA & GONZÁLEZ-MOLERO, 2016; CADIEUX, et al., 2008).

Likewise, antioxidants are a powerful tool in disease prevention and combat oxidative stress in the cells of the body by inhibiting or delaying damage caused by free radicals (OROIAN & ESCRICHE, 2015).

Despite such benefits, these compounds are usually unstable in light, humidity, oxygen, and temperature. In addition, they must withstand acidic stomach conditions and enzymes present throughout the gastrointestinal tract (CHEN et al., 2011; WEN et al., 2017).

One possible approach for the food industry to adapt to this scenario and overcome these challenges is through the development and offer of new functional foods, making microencapsulation a promising alternative. Co-encapsulation is another technique, as it incorporates different functional compounds and allows a single food matrix to present a combination of beneficial and synergistic effects, although this is a novel area and requires further studies regarding its use in developing functional foods.

Therefore, the present literature review aimed to address the development of new functional foods containing different bioactive compounds using different encapsulation techniques in order to overcome the challenges of maintaining the shelf life of these compounds and increase their viability and functionality.

Microencapsulation and Co-encapsulation

Microcapsules are excellent means of introducing bioactive compounds into food or beverages to develop new functional foods. There are different techniques to produce

microcapsules such as physical methods, including freeze-drying, spray drying, solvent evaporation, and precipitation with supercritical fluids. Physical-chemical methods include internal and external ionic gelation, complex coacervation, and liposomes. In addition, microencapsulation can be carried out by chemical methods, such as by molecular inclusion complexation and interfacial polymerization (TYAGI et al., 2011).

Selecting a suitable method to produce microcapsules depends on the desired particle size, the target food, production costs, peculiarities of the material to be encapsulated, wall material, release rate of the active material, etc. (KHADIRAN et al., 2015; ZHU, 2017). The wall or encapsulating material surrounding the nucleus or active material can be comprised of several components, including polysaccharides, lipids, and proteins.

The encapsulation of two or more bioactive compounds into a single matrix is called co-encapsulation (CHEN et al., 2013) (Figure 1). By combining different active compounds, greater viability and bioavailability can be achieved compared to isolated encapsulated elements (ZHANG et al., 2019).

ZAEIM at al. (2019) successfully co-encapsulated probiotics with prebiotics. BAO et al. (2020) also used co-encapsulation to combine the beneficial properties of α-tocopherol and resveratrol into the same matrix. COMUNIAN et al. (2020) described the co-encapsulation of pequi and buriti oils by emulsification and freeze-drying as a promising method to protect carotenoids and increase oxidative stability. HOLKEM et al. (2020) also described the advantages of co-encapsulation to protect probiotics and a proanthocyanidin-rich cinnamon extract against the gastrointestinal tract.

Symbiotic foods

The most commonly used and studied probiotics belong to the genera *Bifidobacterium* and *Lactobacillus*. Probiotic cells bolster the immune system (MORO-GARCÍA et al., 2013), fight pathogens, modulate intestinal flora, reduce lactose intolerance, and have anti-cancer and anti-inflammatory effects (VASILJEVIC & SHAH, 2008; GEORGE KERRY et al., 2018; ALMADA et al., 2015).

Various studies have reported higher microencapsulated probiotic microorganism viability compared to free cells, especially in conditions simulating the gastrointestinal tract (GEBARA et al., 2013; RADDATZ et al 2020). Most commercialized foods that contain probiotic properties are of dairy origin and kept refrigerated (BURGAIN et al., 2011). In this context, new studies have sought to microencapsulate probiotics in different food matrices, such as soy protein bars (CHEN & MUSTAPHA, 2012), green coconut water (BASU et al.,

2018), and acerola nectar (ANTUNES et al., 2013). However, achieving the probiotic effects requires a daily intake of at least 10^6 CFU/g or ml at the time of ingestion (CHAMPAGNE et al., 2011). This is still an obstacle for the development of functional foods containing probiotics in view of their sensitivity to adverse conditions.

Symbiotic microparticles are capable of increasing the survival rates of probiotic microorganisms after exposure to simulated gastrointestinal tract (POLETTO et al., 2019). Among the existing prebiotics, it is possible to highlight inulin, Hi-maize, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), xylooligosaccharides, pectin, lactulose, soy oligosaccharides, and lactitol xylitol (SIRÓ et al., 2008).

In addition to being rich in nutrients, interest in prebiotics increases due to their ability to improve certain organoleptic properties in food (DE MORAIS et al., 2015). The ingestion of prebiotics confers innumerous benefits to human health, such as selectively stimulating probiotic bacteria, modulating mineral absorption, promoting a feeling of satiety, increasing weight loss, reducing the risk of some types of cancer, and improving cholesterol and glucose control (SAAD et al., 2013; POKUSAEVA et al., 2011).

ANEKELLA & ORSAT (2013) developed a symbiotic raspberry juice to evaluate the possible prebiotic effects of maltodextrin as a carbon source. PAIM et al. (2016) analyzed prebiotic jussara juice (*Euterpe edulis* M.) containing *Bifidobacterium spp.* Lactis microencapsulated with different prebiotics (inulin and oligofructose) and maltodextrin and described high probiotic survival rates after encapsulation. GANDOMI et al. (2016) developed apple juice using probiotic capsules and evaluated the effect of adding prebiotic inulin. The authors reported that probiotic survival during storage and passage through the gastrointestinal tract improved with good sensory acceptance of the product.

Furthermore, KRASAEKOOPT & WATCHARAPOKA (2014) observed improved probiotic protection when microencapsulating *Lactobacillus acidophilus* 5 and *Lactobacillus casei* 01 in alginate and chitosan with galacto-oligosaccharides and inulin prebiotics for use in commercial yogurt and orange juice.

Foods containing antioxidants

Antioxidants are powerful tools against degenerative diseases and some types of cancer. These compounds arouse the interest of the food industry as food preservatives since they decrease product deterioration caused by oxidation, in addition to reducing the nutritional loss of the food and improving the conservation of their organoleptic characteristics, pigment,

color, and odor (MARTYSIAK-ZUROVSKA & WENTA, 2012; PISOSCHI & POP, 2015; EROGLU OZKAN et al., 2018).

Some commonly consumed foods, such as fruits, cereals, legumes, and vegetables have antioxidant compounds, however, their consumption alone may not be enough for the necessary intake of antioxidants (YASHIN et al., 2013). In addition, factors such as the presence of light, oxygen, and temperature may cause food degradation, consequently hindering any antioxidant and functional effects. Thus, the use of microencapsulation is an effective alternative to protect antioxidant compounds and their application in new functional foods.

Food antioxidants are categorized into water-soluble compounds, the more notable being phenolic, anthocyanin, flavonoid, citrate, betalain, norbixin, and liposoluble compounds, which include vitamins, carotenes, tocopherols, and terpenoids (CAROCHO et al., 2017). In addition, natural extracts from plants have greater antioxidant capacity compared to synthetic antioxidants (BISWAS et al., 2017).

In the bakery industry, different matrices have already been studied for the application of microcapsules containing antioxidant compounds, for example, a biscuit model with cocoa hulls phenolic extracts (PAPILLO et al., 2018) and pseudocereal-enriched einkorn water biscuits to evaluate the addition of red beetrood pomace extracts (HIDALGO et al., 2018).

In the meat products industry, BALDIN et al. (2018) developed mortadella sausages with a microencapsulated jaboticaba extract. SPINELLI et al. (2016) microencapsulated polyphenols and flavonoids extracted from used grains from a brewery and applied them to fish hamburgers. The authors were able to mask the unpleasant and bitter taste of the extract and achieve greater antioxidant capacity and bioactive content compared to the control sample.

ZOKTI et al. (2016) also used microencapsulation to add a green tea extract to mango juice and found lower degradation rates and greater stability and antioxidant capacity compared to the non-encapsulated material.

New functional foods with the combination of bioactive compounds

Foods or beverages that have a combination of different bioactive sources facilitate the introduction of these compounds as part of the daily diet of consumers. In addition, the constant competitiveness between the food industries leverages interest in the development of innovative products (DE PELSMAEKER et al., 2015).

REID (2002) reported that the combination of probiotic cells with some types of anthocyanins confer synergistic effects on the immunity of the host. Likewise, catechins, which have potential antioxidant activity, improve probiotic survival in foods during their shelf life (SHAH et al., 2010). Furthermore, SU et al. (2008) demonstrated synergistic effects to combat human pathogens by associating green tea extract and probiotic cells. GAUDREAU et al. (2016) successfully co-encapsulated *Lactobacillus helveticus* and green tea extracts by internal emulsification/gelation, although the behavior of the microcapsules in a food matrix was not evaluated.

RIBEIRO et al. (2015) obtained synergistic effects by joining antioxidant extracts of *Suillus luteus* (L.: Fries) (Sl) and *Cooprinopsis atramentaria* (Bull.) (Ca) mushrooms. The authors also co-encapsulated the extracts by spray drying, added them to cottage cheese, and noted greater extract preservation by encapsulation.

Similarly, VAZIRI et al. (2018) mixed *Lactobacillus plantarum* with DHA-rich oil by co-microencapsulation and observed high resistance to simulated gastrointestinal tract conditions. However, the authors did not apply the microparticles to a food matrix.

SHINDE *et al*. (2014) used co-encapsulation to mix *Lactobacillus acidophilus* and an aqueous or ethanolic extract of polyphenol from apple skin and apply it to a model milk drink. The co-encapsulated probiotic with aqueous or ethanolic extract showed the lowest loss of viability compared to the free probiotic and encapsulated probiotic without the extracts after 50 d at 4 °C. The co-encapsulation of probiotic bacteria has also been studied in combination with omega-3 (ERATTE et al., 2017). A probiotic petit suisse cheese added with non-encapsulated jabuticaba skin or bark extract also showed high anti-toxicity activity and probiotic survival, however, the behavior and viability of these compounds in the gastrointestinal tract were not evaluated (PEREIRA et al., 2016a; PEREIRA et al., 2016b).

CHAIKHAM (2015) increased the survival of probiotic cells by co-encapsulating with a cashew extract, green tea extract, and adding the product to fruit juices (blackberry, maoberry, longan, and melon) and stirred yogurt.

Challenges and effectiveness of applying bioactive compounds in the development of new functional foods

Microencapsulating active compounds is an effective means of meeting the constant changes of the consumer market, allowing the food industry to create products with functional and nutritional appeal. However, the industry still faces several challenges in order for these compounds to remain viable throughout the storage period and when ingested.

Determining the correct wall material is essential for successful microencapsulation, as it will influence the size, shape, viscosity, and even the stability of the microcapsules during storage. Moreover, the wall material must protect the nucleus from unfavorable conditions and mask unwanted sensory aspects that certain bioactive compounds tend to present (WEINBRECK et al., 2010).

Sensory evaluations are an important factor when analyzing the acceptance of a new food product, as microcapsule sizes can range from 0.2 to 5000 µm (SILVA et al., 2014). To prevent the microcapsules from being sensorially perceived, which results in poor acceptance by the consumer, choosing products with adequate physical parameters (viscosity, consistency, and texture) and capable of masking the microcapsules may be an alternative.

When inserting microcapsules containing bioactive substances into food, aspects of the food matrix itself may be limiting factors for the prolonged stability of the active compound, in addition to the conditions in which they are stored in, including the presence of light and/or high temperatures. Further research must evaluate the effectiveness of foods containing microcapsules throughout their shelf life in order to comply with their claims and provide the declared functional effects when consumed. Finally, the functional food must be resistant to the intestinal tract in order to provide health benefits, with the microcapsules being released only at their place of action (JYOTHI et al., 2010). Simulating the passage through the gastrointestinal tract and *in vivo* simulations may be alternatives for this, as it is possible to verify if the microcapsules keep the active compound viable and bioavailable after contact with bile salts and low pH. Thus, the authors believe that only by considering such aspects can the challenges of incorporating one or more functional compounds into food be overcome.

CONCLUSION

Microencapsulation and co-encapsulation of bioactive substances are promising methodologies, providing positive results and meeting the technological challenge of maintaining compound stability and viability. Moreover, these alternatives enable the development of new food products, increase the range options for the consuming public, and promote greater nutritional values and therapeutic benefits with lower processing costs for product development. Nevertheless, further studies are necessary, especially regarding *in vivo* application. In addition, microencapsulation and co-encapsulation behavior and stability in different food matrices and their effects should be evaluated and proven through trials and

research while aiming to improve the well-being of the population and fight diseases in a practical and healthy way.

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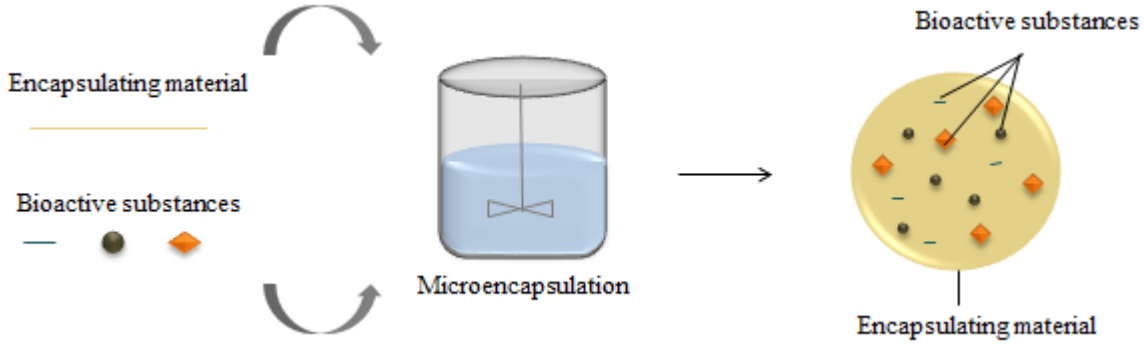

Figure 1. Use of microencapsulation for the union of different active compounds. Adapted from: CHAWDA et al., (2017).

5.3 ARTIGO II –

VIABILITY AND STABILITY EVALUATION OF *Lactobacillus casei* LC03 CO-ENCAPSULATED WITH RED ONION (*Allium cepa* L.) PEEL EXTRACT

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Viability and stability evaluation of *Lactobacillus casei* LC03 co-encapsulated with red onion (*Allium cepa* L.) peel extract

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Abstract

This study aimed to evaluate the effects of adding different concentrations (5, 20, and 40%) of red onion (*Allium cepa* L.) peel extract to probiotic microparticles on the viability and stability of a *Lactobacillus casei* LC03 probiotic. The survival of the probiotic under simulated gastrointestinal conditions, its viability in storage, and the resistance of the microparticles to thermal treatments were evaluated, as well as the morphology, size distribution, and encapsulation efficiency of probiotics and compounds in the extract. All microparticle formulations showed high probiotic retention efficiency (>90%). The sizes of the microparticles ranged from 149.29 ± 1.67 to 167.05 ± 3.88 µm. The treatment containing alginate + 20% extract increased probiotic survival under simulated gastrointestinal conditions than the other treatments. It was possible to increase the heat resistance of encapsulated probiotics over free microorganisms. During storage at 25°C, the alginate

microparticles + 5 and + 20% extract stood out, whereas the most prolonged period of viability was reached by alginate alone and alginate microparticles + 5% extract at 7°C. At -18°C, all microparticle formulations remained stable during 90 days of storage. These findings suggest that such microparticles can promote the extended viability and stability of the probiotic *L. casei* under different conditions.

Keywords: alginate, probiotics**,** co-encapsulation, gastrointestinal tract, heat resistence

1. Introduction

As an alternative to ingesting probiotic cells, the food industry has paid increasing attention to research focused on developing products that contain these microorganisms in their formulations. The co-encapsulation method protects probiotics by involving them in a secondary coating to maintain their viability and functionality due to interaction with adverse factors during their shelf life. This technique also allows connecting different compounds, and the possible benefits of these compounds for the probiotic cultures can be measured. A co-microparticle is obtained by joining two or more bioactive compounds into a single matrix using the microencapsulation method (Chen, McGillivray, Wen, Zhong, & Quek, 2013).

Applying bioactive extracts to probiotic particles is still sparsely studied. Chaikham (2015) researched the impacts of Thai herbal extracts on the stability of probiotics in alginate particles. The authors chose compounds with high levels of bioactive compounds and high antioxidant activity and managed to improve the viability of probiotics with cashew flower and green tea extract. Similarly, co-encapsulating bioactive compounds from blackberry juice with *Lactobacillus acidophilus* by spray drying was tested by Colín-Cruz, Pimentel-González, Carrillo-Navas, Alvarez-Ramírez, & Guadarrama-Lezama (2019), who obtained good viability of probiotics after storage when using whey protein as a wall material. However, more studies are needed to evaluate how different bioactive extracts can act on the cell viability of probiotics.

Onions (*Allium cepa* L.) are a good source of bioactive compounds with pharmacological and therapeutic properties that include organosulfur compounds, phenolic acids, thiosulfinates, and flavonoids, especially quercetin and anthocyanins, being the latter found exclusively in red onions (Zeng et al., 2017). These compounds act by preventing several types of cancer, including bladder, prostate, esophagus, and stomach cancer (Jan et al., 2010). Furthermore, Desjardins (2008) noted that several studies reported that onions and their relatives may act against cardiovascular diseases, diabetes, asthma, and produce

prebiotic effects. Quercetin has been reported to benefit the micro-ecological balance of intestinal flora (Zhang et al., 2014). It is also known that higher levels of these compounds are found in onion peels than other parts of this and other vegetables and fruits (Moon, Do, Kim, & Shin, 2013). The peels, however, are commonly disregarded upon processing/consuming the vegetable, despite possessing significant antioxidant activity that protects against cellular damage caused by reactive oxygen species (produced by cellular metabolism and other exogenous environmental agents) capable of oxidizing cellular biomolecules (nucleic acids, proteins, lipids, carbohydrates, and enzymes) (Prior, Wu, & Schaich, 2005). Thus, it is essential to add value to this residue, which is extremely rich in active compounds, avoiding its disposal through formulations that allow its insertion into food matrices while seeking to inhibit its undesirable sensory effects due to its characteristic odor and flavor.

Given the above, this study aimed to develop microparticles and investigate the effects of co-encapsulating alginate (ALG) with red onion peel extracts at different concentrations on the stability and viability of the probiotic *L. casei* LC03.

1. Materials and Methods

2.1 Materials

Sodium alginate (Protanal, Brazil), calcium chloride (Vetec Química Fina, Rio de Janeiro, Brazil), and probiotic culture *L. casei* LC 03 (Coana, Brazil) were used to produce the particles, in addition to pepsin, Folin-Ciocalteu reagent, sodium carbonate (Dinâmica, São Paulo, Brazil), pancreatin, and bovine bile (Sigma Aldrich, Brazil).

2.2 Methods

2.2.1 Inoculum preparation

The probiotic culture *L. casei* LC 03 (1 g) was activated anaerobically in 100 mL of MRS broth (Himedia) for 15 to 18 h at 37 ± 1°C. It was then centrifuged at 4670 × g for 15 min at 4 ± 1°C and washed with saline (0.85g/100mL).

2.3 Red onion peel extract collection

The extracts were prepared according to the method of Viera et al. (2017) with adaptations. The sanitized samples (sodium hypochlorite 200 mg L^{-1}/20 min) had their peels removed and dried in an oven with forced air circulation (Marconi, MA-035/100, Piracicaba,

Brazil) at 50 ± 1°C for 24 h. Next, the samples were ground in a knife mill (Mill MA 630/1-Marconi; 5 rpm for 10 s). A red onion peel sample (3 g) was added to 60 mL of 80% ethanol and kept under stirring at 80 rpm using a mechanical stirrer for 120 min at 25 ± 2°C to collect the extracts. The extracts were then rotary-evaporated, and their volume was adjusted with distilled water for further use.

2.4 Reducing capacity

A sample (0.4 mL) was added to 2 mL of Folin-Ciocalteu 2 mol/L reagent (1:10) for 5 min to evaluate the reducing capacity of the extract. Then, 1.6 mL of sodium carbonate (Na_2CO_3; 7.5g/100mL) was added, and the sample was placed in a water bath at 50 ± 2°C for 5 min. The absorbance obtained in the reaction were read in a spectrophotometer at a wavelength of 765 nm. A standard curve was prepared with gallic acid to quantify the reducing capacity (a mechanism based on electron transfer), and the results were presented as mg of gallic acid equivalent (GAE) per g sample (mg GAE/g) (Roesler et al., 2007).

2.5 Flavonoid content determination

A sample (0.5 mL) received 150 µL of $NaNO_2$ (5g/100mL) in a test tube for 5 min to determine the flavonoid content. Next, 150 µL of $AlCl_3$ (10g/100mL) was added, and the sample was allowed to stand for another 6 min protected from light. Afterward, 1 mL of NaOH (1 mol/L) was added, followed by distilled water (1.2 mL). The absorbance obtained in the reaction were read in a spectrophotometer at a wavelength of 510 nm. A standard curve of quercetin was prepared to determine the flavonoid content, and the results were presented as mg quercetin equivalent (QE) per g sample (mg QE/g) (Zhishen, Mengcheng, & Jianming, 1999).

2.6 Total monomeric anthocyanin content determination

The anthocyanin content was determined by the differential pH method (Giusti; Wrolstad, 2001). The extract sample (0.5 mL) was diluted separately with 2.5 mL of 0.025 mol/L potassium chloride buffer solution (pH 1.0) and 0.4 mol/L sodium acetate buffer solution (pH 4.5). The diluted solutions were then left at room temperature for 15 min, and the absorbance of each dilution was read at the wavelengths of 520 and 700 nm. The anthocyanin content was calculated using the following equation:

Anthocyanin content (mg/100 g dry matter) = A x MW x DF ⁄ (ε x W)

Where A is the absorbance (A_{520nm} - A_{700nm}) $pH_{1.0}$ - (A_{520nm} - A_{700nm}) $pH_{4.5}$, MW is the molecular weight of cyanidin-3glucoside ($C_{15}H_{11}O_6$, 449.2), DF is the dilution factor, ε is the molar absorptivity (26900), and W is the sample weight (g).

2.7 Antioxidant capacity

The oxygen radical absorbance capacity (ORAC) was analysed as proposed by Ou et al. (2001), and the results were expressed in μmol Trolox equivalents per gram of red onion peel (μmol Trolox/g).

2.8 Microparticle production - external ionic gelation technique containing *L. casei* and red onion peel extract

Microencapsulation by external ionic gelation was performed according to Etchepare et al. (2016) with adaptations. A mixture of alginate (2g/100 mL), probiotic culture *L. casei* LC 03, and red onion peel extract was sprayed 12 cm apart in 0.1 M $CaCl_2$ by a dual-fluid atomizer nozzle (0.3 mm) at 0.125 kgf/cm. Afterward, the microparticles were stirred for 30 min in the $CaCl_2$ solution and sieved (0.038 mm, 400 mesh). The different formulations of the microparticles are listed in Table 1.

2.9 Microparticle characterization

2.9.1 Optical microscopy and average size

Microparticle morphology was evaluated using an optical microscope (Carl Zeiss Axio Scope A1, Oberkochen, Germany) equipped with an Axio Cam MRc digital camera (Carl Zeiss). The particle size distribution was determined on a Mastersizer 2000 laser diffraction particle size analyzer (Malvern, UK) with water as the dispersion medium. The results reflect the average diameter of the analyzed microparticles (D [4: 3]) resulting from five repetitions performed by the equipment.

2.10 Viable cell count

The bacteria were released from the microparticles according to Sheu, Marshall, & Heymann (1993) with modifications. A sample (1 g) was added to 9 mL of sterile phosphate buffer solution (pH 7.5) and homogenized for 10 min to release the probiotic (Figure 1E).

Subsequently, serial decimal dilutions were prepared in peptone water (0.1g/100mL) with 1.0 mL plating of the dilutions and in triplicate for sterile Petri dishes, then added with MRS Agar. The plates were then incubated at 37 ± 1°C for 72 h under anaerobic conditions.

2.11 Encapsulation efficiency of polyphenols and flavonoids

Particle breaking was performed according to Robert et al. (2010) with adaptations. For this, a solution containing 0.5 mL acetonitrile and 0.5 mL methanol:acetic acid:water (50:8:42 mL/mL//mL) was added to 1 g of microparticles. This mixture was vortexed for 4 min and sonicated for 60 min. Afterward, the supernatant was collected and centrifuged at 5000 rpm for 15 min. From this liquid, the encapsulation efficiency of polyphenols, flavonoids and total monomeric anthocyanins was determined according to the method described in sections 2.4, 2.5 and 2.6, respectively, by calculating the percentage of encapsulated polyphenols and flavonoids over the total of these compounds in the initial alginate solutions.

2.12 Encapsulation efficiency of the probiotics

The encapsulation efficiency (EE%) of the probiotics was calculated as proposed by Martin, Lara-Villoslada, Ruiz, & Morales (2013) in Eq. 1.

$$\text{Eq. 1. } EE = (N/N0) \times 100$$

Where N is the number of viable cells (log CFU g^{-1}) released by breaking microparticles and N0 is the number of viable cells (log CFU g^{-1}) in the cell concentrate before microencapsulation.

2.13 Evaluation of microparticles under simulated gastrointestinal conditions

The method proposed by Madureira, Amorim, Gomes, Pintado, & Malcata (2011) with adaptations was employed to evaluate the particle resistance against simulated gastrointestinal conditions. The bacteria in the different microparticle treatments and pure culture were subjected to simulated esophagus/stomach conditions (pH 2.0/90 min in the presence of pepsin), duodenum conditions (pH 5.0/20 min in the presence of pancreatin and bile salts), and ileum conditions (pH 7.5/90 min) in a TE 421 shaker (Tecnal, Piracicaba, São Paulo, Brazil) at 37 ± 2°C. Aliquots were collected and plated, according to item 2.10, at the end of each step of the gastrointestinal simulation.

2.14 Microparticle resistance to heat treatment

The free and encapsulated microparticles (1 g), along with peptone water (9 ml), were subjected to 72 ± 1°C for 15 s and 63 ± 1°C for 30 min, then immersed in ice for cooling. Next, aliquots were collected and plated according to item 2.10 (Zhang, Lin, & Zhong, 2015).

2.15 Microparticle evaluation under different storage conditions

Counts were performed every 15 days according to item 2.10, with the samples stored in sterile Eppendorf tubes and protected from light at 25 ± 1°C, 7 ± 1 °C, and -18 ± 1°C, thus simulating room temperature, refrigeration, and freezing conditions, respectively, for 90 days.

2.16 Statistical analysis

Data were submitted to analysis of variance (ANOVA) using Statistica 7.0 (2004, Statsoft Inc., Tulsa, OK, USA), followed by Tukey's test for comparison of means, considering a 5% significance level ($p<0.05$). Tests were performed in triplicate, and data expressed as mean ± standard deviation.

2. Results and discussion

3.1 Microparticle characteristics: shape, size, and encapsulation efficiency

The images of the microparticles produced with different encapsulating matrices containing *L. casei* LC03 are shown in Figure 1. The microparticles had similar morphology regardless of the treatment, with spherical shapes and smooth surfaces in every formulation. Optical microscopy revealed that the probiotics were successfully encapsulated, and it was possible to observe them distributed throughout the microparticle matrix (Figure 1A).

The diameters of the different microparticle formulations are listed in Table 2. To be classified as such, a microparticle must have a size ranging from 0.2 to 5000 µm (Favaro-Trindade, Pinho, & Rocha, 2008). In the external ionic gelation technique, factors such as composition, concentration of wall material, nozzle diameter, and distance between the extruder nozzle and gelling solution directly influence the particle size to be obtained (Anal, Bhopatkar, Tokura, Tamura, & Stevens, 2003). Nevertheless, this technique is well known for producing microparticles with spherical shapes and uniform sizes (Đorđević et al., 2014). Smaller particle sizes provide better homogeneity and distribution of the active compound in the product while avoiding undesirable effects on food texture (Chitprasert, Sudsai, &

Rodklongtan, 2012; Shaharuddin & Muhamad, 2015). The mean microparticle sizes were between 149.29 ± 1.67 and 167.05 ± 3.88 μm. These results are similar to those found by Araujo Etchepare et al. (2020), who obtained probiotic microparticles with sizes ranging from 107 to 222 μm using the external ionic gelation technique. Similarly, Poletto et al. (2019a) developed microparticles ranging from 127.5 to 234.6 μm by external ionic gelation followed by freeze-drying.

Moreover, adding the extract influenced the average particle diameter, as those particles were significantly smaller ($p<0.05$) than the others only containing alginate. It is known that the constitution of the encapsulating material influences the size and shape of the capsules (Sandoval-Castilla et al., 2010). When using only alginate, its increased concentration allows more points for binding with Ca^{2+}, which increases the alginate filament network and generates a more consistent and massive matrix (Wang et al., 2013). In contrast, Vodnar & Socaciu (2014) used the extrusion method and developed chitosan-coated alginate particles containing the probiotic *Lactobacillus plantarum* or *L. casei* with and without adding selenium-enriched green tea. The authors observed that the microparticles containing selenium-enriched green tea and LC had larger diameters than those only containing the probiotic.

The results of probiotic encapsulation efficiency in microparticles with and without the extract are listed in Table 2. The encapsulation technique needs to present a high retention rate of the active compound to effectively reach its site of action in high quantities. When evaluating the pure extract aspects, its reducing capacity was 785.84 ± 26.56 mg/g, the number of flavonoids was 67.13 ± 1.15 mg/g, the content of total monomeric anthocyanins was 53.92 ± 2.30 mg/g, and the antioxidant capacity was 1096.45 μmol Trolox/g (data not shown). Adding red onion peel extracts to the capsules did not produce any significant changes in probiotic viability during the encapsulation process, as there was high probiotic retention in the capsule (>90%) in all formulations.

The external ionic gelation technique may have directly affected the encapsulation efficiency obtained in this study, as it allowed particle development without extreme temperatures or organic solvents. These results corroborate Poletto et al. (2019b) and Vaziri, Alemzadeh, Vossoughi, & Khorasani (2018), who obtained encapsulation efficiencies of 96.75 and 99.9%, respectively, when using extrusion/external ionic gelation techniques to encapsulate probiotic cells. Other researchers did not obtain such satisfactory EE% results when joining probiotics and bioactive extracts through microencapsulation compared to the data obtained here. Vodnar & Socaciu (2012) co-encapsulated bifidobacteria with green tea

extracts using alginate and achieved EE% of the probiotic equivalent to 37.5%. Similarly, Chávarri et al. (2010) obtained low EE% when co-encapsulating *Lactobacillus gasseri* (19.5%) and *Bifidobacterium bifidum* (22.2%) with quercetin (prebiotic), attributing these findings to the interaction between the flavonoid and probiotic cells. From these different results, the importance of evaluating the best concentration of extract/compound to be added in microparticles containing probiotics is noted, since each compound contains its particularities and contents of phenolic compounds, flavonoids, anthocyanins, antioxidant activity, among others , which can influence probiotics both positively and negatively.

The encapsulation efficiency of reducing compounds, flavonoids and total monomeric anthocyanins in the microparticles containing red onion peel extracts (A5, A20, and A40) are detailed in Table 2. Microparticles containing 5% extract (A5) showed the highest retention rate of reducing compounds after microencapsulation (84.77%). Meanwhile, treatments A20 and A40 presented retention rates of 69.85 and 68.44%, respectively. Our findings reached more satisfactory values than those obtained by Gaudreau, Champagne, Remondetto, Gomaa, & Subirade (2016), who acquired polyphenol EE% ranging from 36.1 to 79.0% when co-encapsulating *Lactobacillus helveticus* with a green tea extract at concentrations of 500-2000 µg/mL. The authors also reported higher polyphenol EE% at the lowest extract concentration (500 µg/mL). As for the flavonoid and total monomeric anthocyanins content in the particles, the highest EE% was achieved in treatment A20 (65.96% and 61.25% respectively).

3.2 Survival of free and microencapsulated *L. casei* exposed to simulated gastrointestinal conditions

Potential of hydrogen (pH) is a crucial factor that can limit the survival rate of probiotics, especially at low values (Sathyabama, Ranjith Kumar, Bruntha Devi, Vijayabharathi, & Brindha Priyadharisini, 2014). To exert their beneficial effects on the host, many of these microorganisms must reach the colon and survive the passage through the stomach and duodenum. Nonetheless, several studies have described that the probiotic *L. casei* is sensitive to acidic conditions, which leads to a reduction in its viability under gastrointestinal tract conditions (Dimitrellou et al., 2016; Dimitrellou et al., 2019). Hence, probiotics in free and microencapsulated treatments, with (A5, A20, and A40) and without (ALG) extracts, were subjected to simulated esophagus/stomach, duodenum, and ileum conditions to resemble the gastrointestinal tract (Figure 2).

At the beginning of the analysis, the free probiotic had a count above 10 log CFU g^{-1}, whereas the probiotic encapsulated in microparticles had a count close to 9 log CFU g^{-1} across

the different treatments. After 90 min of contact with simulated gastric juice (pH 2.0), the free probiotic underwent a reduction of 3.98 log CFU g^{-1}, suggesting that protective material is necessary. Conversely, encapsulation with alginate and alginate + extract provided noteworthy protection in simulated gastric juice (Figure 2). More specifically, the total of probiotics released from inside the particles was only 3.79, 3.03, 3.93, and 4.38 log units for ALG, A5, A20, and A40, respectively.

In simulated duodenum conditions (pH 5.0/20 min), the free probiotic showed an even higher loss of viability (4.41 log CFU g^{-1}) than in the stomach/esophagus. Furthermore, in this section of the simulation, the free probiotic reached viability below the recommended to promote beneficial effects (above 6 log CFU g^{-1}). Damage to the probiotic cell wall may occur in the presence of bile salts, leading to cell disruption and death (Bron et al., 2004). Nevertheless, the encapsulation process proved positive in protecting the probiotics because there was no full cell release inside the particles, thus they remained stable.

Alginate microparticles are designed so that the release of the active compound occurs only at a pH close to 7.0 (ileum), since this material is only dissolved in this pH range. However, the high porosity of alginate can lead to disadvantages in the elaboration of probiotic microparticles, with reduced viability during storage and ingestion (Chávarri et al., 2010). For this, different materials can be used to block these pores, through the formation of a protective layer, such as, for example, chitosan, polyamino acids (for example, polylysine) and whey proteins (Anjani et al., 2007; Gombotz et al., 1998). Despite this, Angelica Andrade Lopes. (2020) reported that coating alginate microcapsules with chitosan did not improve the survival of probiotics to in vitro digestion.

In this study, alginate alone (ALG) was able to maintain probiotic viability in the face of low pH and bile salts, in addition, the onion peel extract resulted in an improvement in the survival of probiotics under these same conditions, possibly by exerting its prebiotic effect. Prebiotic compounds, in general, are able to increase the survival of probiotic bacteria in the passage through the gastrointestinal tract, as they act as substrates used by these microorganisms, resulting in synergistic effects (Ballan , Battistini, Xavier-Santos & Saad, 2020; Sathyabama, Ranjith kumar, Bruntha devi, Vijayabharathi & Brindha priyadharisini, 2014).

After 200 min of analysis (pH 7.5), the microparticles of the different treatments (ALG, A5, A20, and A40) disintegrated, resulting in cell release at the desired site of action (Figure 1F). When comparing the loss of viability of the probiotic *L. casei* in free and encapsulated forms, the latter reached its site of action in the required amounts (above 6 log

CFU g^{-1}) in all microparticle formulations. Notwithstanding, the former lost its viability in the duodenum section, failing to reach the ileum in the required amount to produce beneficial effects, demonstrating that this microorganism demands a barrier against external factors.

Similar to these findings, other microencapsulation techniques, including spray-drying (Dimitrellou et al., 2016), emulsification (Mandal, Puniya, & Singh, 2006), and freeze-drying (Li et al., 2019), also proved to be effective in preserving LC viability compared to free cells under simulated gastrointestinal conditions. Using an external ionic gelation/extrusion technique, Farias et al. (2019) found that microencapsulation also exerted protective effects for *Lactobacillus rhamnosus* ASCC 290 and LC ATCC 334 added to ice cream during simulated gastrointestinal conditions.

Comparison of the microparticles with the extracts (A5, A20, and A40) and without ALG showed that treatments A5 and A20 exhibited improved probiotic viability. However, A40 had the most significant reduction in probiotic viability at the end of the simulation, implying that higher concentrations of red onion extracts tend to be less favorable for probiotic viability in the simulated gastrointestinal tract. Even so, all the formulations reached the ileum in the required amounts. The ability of microparticles to protect the active compound in the stomach and promote its release in the ileum has also been cited by several researchers (Raddatz et al., 2020; Coelho-Rocha et al., 2018; da Silva et al., 2019). In addition, phenolic compounds have already been reported to promote the stimulation of probiotic microorganisms, balancing the intestinal flora (Li et al., 2016) as well as acting as prebiotics (Lee, Jenner, Low, & Lee, 2006), which may have led to increased probiotic survival under the simulated gastrointestinal tract conditions evaluated herein.

3.3 Resistance of free and microencapsulated *L. casei* to heat

The free and microencapsulated probiotics went through the processes of fast and slow pasteurization (72 ± 1°C for 15 s and 63 ± 1°C for 30 min, respectively) to evaluate the protective effects of the microparticles against high temperatures, since these processes are used as a fundamental step to produce various foods and beverages (Table 3). At 72 ± 1°C for 15 s, the treatment that presented the highest heat resistance was A5, with a reduction of 1.29 log CFU g^{-1} from its initial count. Additionally, it was possible to note an improvement in cell viability (less reduction) in the treatments that contained the extracts (A5, A20, and A40) after heating as opposed to the free probiotic and the microparticles without the extract (ALG).

As in the previous condition (63 ± 1°C for 30 min), treatment A5 showed a lower reduction in cell viability (0.79 log CFU g^{-1}) than the other microparticle formulations and the

free probiotic. Treatments ALG and A20 also maintained high cell viability, with reductions of only 1.58 and 1.33 log CFU g^{-1}, respectively, from their initial counts. Sodium alginate combined with Ca^{2+} ions formed an extremely thermostable gel, which may have contributed to its high stability under the elevated temperatures (de Vos et al., 2009). Furthermore, compounds such as anthocyanins can be preserved against heat treatments through microencapsulation (Yamashita et al., 2017). The free microorganism and microparticles of A40 did not show the same satisfactory resistance, with higher reductions (1.72 and 2.34 log CFU g^{-1}, respectively) than the other treatments.

These results corroborate Rather, Akhter, Masoodi, Gani, & Wani (2017), who developed microcapsules of *L. plantarum* NCDC201 and LC NCDC297 in double alginate coatings by extrusion and observed that the microcapsules provided better thermal protection to the probiotics (75ºC for 1 and 10 min), resulting in significantly higher cell survival compared to free cells.

By comparing the two types of pasteurization evaluated in this study, significant alterations in the different microparticle formulations were only observed in A5, which maintained better viability by slow pasteurization, and A40, where higher viability was achieved by fast pasteurization.

3.4 Free and microencapsulated *L. casei* viability under different storage conditions

The biggest challenge in incorporating probiotics into food matrices is maintaining their viability and stability during processing, storage, and product consumption (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). The literature reveals that probiotics have low viability when added to foods in their free form (de Vos, Faas, Spasojevic, & Sikkema, 2010), which is limiting to develop functional matrices since consumers are recommended to ingest 10^6–10^9 CFU/g or mL of viable probiotics per day to stay healthy.

The content of encapsulated and free *L. casei* LC03 stored for 90 days at room temperature (25 ± 1°C), refrigeration (7 ± 1°C), and freezing (-18 ± 1°C) was determined (Table 4) in order to evaluate the suitability of encapsulated cells for food industry applications. Under room temperature conditions (25 ± 1°C), treatments A5 and A20 stood out, maintaining probiotic viability for a prolonged period of 60 days. The ALG treatment maintained viability for 45 days and A40 for 15 days. The free probiotics also maintained high cell counts at this temperature. In another study, Witzler, Pinto, Font de Valdez, de Castro, & Cavallini (2017) did not achieve the same results when using the extrusion method

followed by freeze-drying. The probiotic *Enterococcus faecium* CRL 183 remained viable for merely 14 days in capsules under storage at 25.0 ± 2°C.

Under higher temperature conditions, there may also be increased degradation of substances such as phenolic compounds, which tend to be more sensitive to heat (de Souza et al., 2019). According to the literature, the progression of biomass composed of fungi can be prevented using phenolic compounds (Porte & Godoy, 2001), which may help maintain the shelf life of capsules at this temperature.

During storage at 7 ± 1°C, there was a loss of viability in the free microorganism and A40 after 15 days of storage. Nonetheless, A20 extended the shelf life of the probiotic by ~60 days. The ALG and A5 stood out even more by achieving cell viability above the recommended at the end of 90 days of storage. These satisfactory results are because of the deterioration of microorganisms and the capsules in the presence of high humidity occurring more slowly at lower temperatures (Abe, Miyauchi, Uchijima, Yaeshima, & Iwatsuki, 2009).

Under freezing conditions (-18 ± 1°C), all particle formulations (ALG, A5, A20, and A40) and the free probiotics maintained their cell viability until the end of 90 days of storage (Table 4). However, when evaluating only the microencapsulated probiotic in the four different treatments, it was possible to observe that the ALG, A5 and A20 formulations had the highest cell counts after 90 days (7.65 ± 0.11, 8.01 ± 0.06 and 7.80 ± 0.03 log CFU g^{-1}, respectively) without significant differences ($p > 0.05$) between them, while the A40 formulation had lower cell counts (6.05 ± 0.28 log CFU g^{-1}), with this it is possible to observe that the A40 microparticles did not present as satisfactory performance as the others. At negative temperatures (e.g., -18°C), the metabolic activity of probiotic microorganisms decreased, which may explain the high cell viability of the free and microencapsulated probiotics in all treatments. Furthermore, Reale et al. (2015) observed that the LC strain is more resistant against temperature changes compared to other probiotics, including *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. Xu, Gagné-Bourque, Dumont, & Jabaji (2016) evaluated the shelf life of LC ATCC 393, free for 35 days and encapsulated by extrusion followed by freeze-drying for 84 days, at 22, 4, and -15 °C and noted that the best probiotic viability was obtained at -15°C, as also demonstrated here. For the free microorganism, its survival rate was higher at 4°C, albeit it also remained viable after 35 days at -15°C.

Further studies on the mechanism of action of extracts on probiotic microorganisms are needed. Still, a possible explanation for the satisfactory performance of A5 and A20 in increasing probiotic viability under the conditions evaluated (room, refrigeration, and freezing

temperatures) is the ability of the active compounds to act as antioxidants, anti-radicals, and prebiotics during the storage period. At higher extract concentrations (i.e., A40), the probiotics either had lower counts compared to the other treatments (at -18°C) or lost viability before the other treatments (at 25 and 7°C) during storage, suggesting that the active compounds present in the extract had adverse effects on the microorganisms. Thus, from these data, extract concentrations of 5 and 20% added to alginate microparticles by extrusion can increase the viability of the probiotic LC during storage under different temperature conditions.

4. Conclusion

Microencapsulation proved to be an effective alternative for the safe and efficient delivery of the probiotic *Lactobacillus casei* LC03. Red onion peel extracts were successfully inserted into the microparticles and helped increase probiotic viability. Nevertheless, the concentration of the extract added to the microparticle must be considered. Low concentrations of the evaluated extract performed better in aiding the survival of the probiotic under different temperature, gastrointestinal, and storage conditions. In contrast, high concentrations may impair its viability under the same circumstances, considering the antimicrobial potential of the extract. Thus, at concentrations below 40%, the extract allows producing a microparticle that combines different active compounds with positive effects on its viability. As to its application, a longer period of probiotic viability was observed at lower temperatures (7 ± 1 and -18 ± 1°C); nonetheless, the application will depend on the matrix to be developed and on its shelf life. Therefore, these microparticles can be assigned to matrices at room temperature. Thus, the use of red onion peel extract is a viable alternative to ensure the viability of the probiotic *L.casei* in alginate microparticles obtained by internal ionic gelation.

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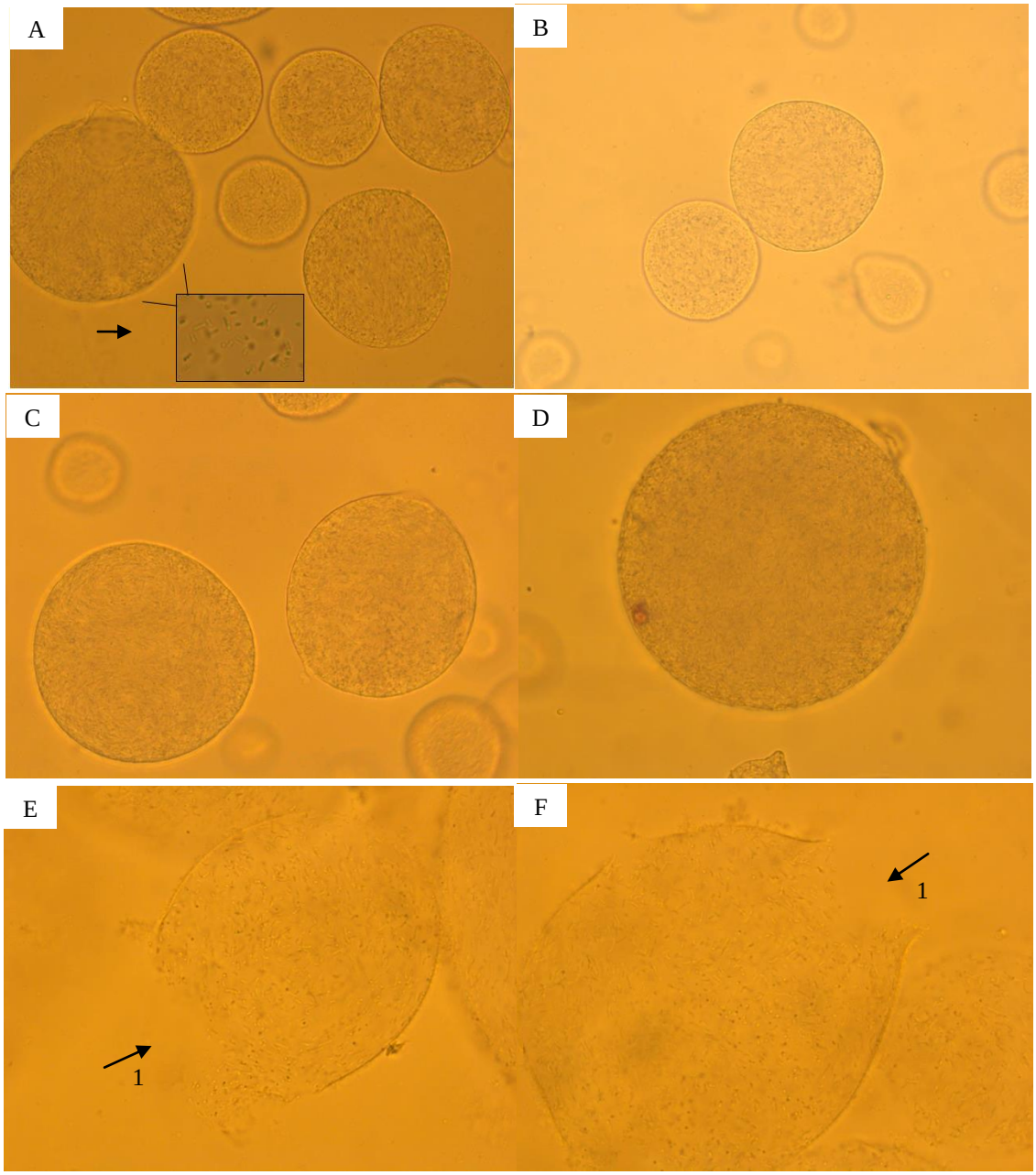

Fig 1. Optical microscopy of alginate microparticles with different concentrations of red onion peel extract containing *L. casei* obtained by external ion gelation. A = 2% alginate microparticles (ALG) (40x), where number 1 shows an enlargement of the microorganisms present inside the alginate microparticle (100x); B = 2% alginate microparticles + 5% extract (A5) (40x); C = 2% alginate microparticles + 20% extract (A20) (40x); D = 2% alginate microparticles + 40% extract (A40) (40x). E = alginate microparticles, where the number 1 shows a break in the microcapsule in the buffer solution 7.5 (40 ×); F = alginate microparticles, where the number 1 shows a break in the microcapsule during simulated ileum conditions at pH 7.5 and 37 ° C.

Table 1. Composition of different encapsulating matrices (ALG, A5, A20 and A40) produced by external ionic gelation containing *L. casei* and red onion peel extract

Treatment	***Composition***
ALG	Particles of alginate (2g/100mL) + *L. casei*
A5	Particles of alginate (2g/100mL) + *L. casei* + 5% red onion peel extract
A20	Particles of alginate (2g/100mL) + *L. casei* + 20% red onion peel extract
A40	Particles of alginate (2g/100mL) + *L. casei* + 40% red onion peel extract

Table 2 – Characteristics of particles containing *L. casei* obtained by external ionic gelation with alginate and red onion peel extract in different concentrations.

Treatment	Particle size (µm)	EE% probiotics	EE% reducing compounds	EE% flavonoids	EE% total monomeric anthocyanins
ALG	167.05 ± 3.88^{a}	93.04 ± 0.72^{ab}	-----	-----	-----
A5	159.40 ± 4.25^{b}	92.28 ± 0.08^{b}	84.77 ± 0.01^{a}	33.29 ± 0.00^{c}	38.27 ± 0.93^{c}
A20	149.29 ± 1.67^{c}	92.97 ± 0.57^{ab}	69.85 ± 0.08^{b}	65.96 ± 0.02^{a}	61.25 ± 1.06^{a}
A40	153.81 ± 2.65^{bc}	93.95 ± 0.15^{a}	68.44 ± 0.36^{b}	49.15 ± 0.09^{b}	47.49 ± 0.40^{b}

ALG = 2% alginate particles; A5 = 2% alginate particles + 5% extract; A20 = 2% alginate particles + 20% extract; A40 = 2% alginate particles + 40% extract. EE% = encapsulation efficiency. Means followed by the same lower case letter in the column do not differ statistically from each other by the Tukey test, with a significance of 5%. Means found in triplicate. Different lowercase letters on columns (a–c) indicate significant differences between formulations ($p < 0.05$).

Table 3 – Thermal resistance of particles containing *L. casei* obtained by external ionic gelation with alginate and red onion peel extract in different concentrations.

	Free culture	**ALG**	**A5**	**A20**	**A40**
Initial	10.13 ± 0.04^{aA}	9.43 ± 0.07^{aB}	9.39 ± 0.05^{aB}	9.42 ± 0.05^{aB}	9.52 ± 0.01^{aB}
72 °C / 15 s	8.63 ± 0.05^{bA}	$7,80 \pm 0.18^{bB}$	8.10 ± 0.10^{cB}	7.96 ± 0.27^{bB}	8.05 ± 0.13^{bB}
63 °C / 30 min	8.41 ± 0.39^{bA}	7.85 ± 0.13^{bB}	8.60 ± 0.12^{bA}	8.09 ± 0.06^{bAB}	7.18 ± 0.06^{cC}

ALG = 2% alginate particles; A5 = 2% alginate particles + 5% extract; A20 = 2% alginate particles + 20% extract; A40 = 2% alginate particles + 40% extract. EE% = encapsulation efficiency. Means followed by the same lowercase letter in the column and uppercase in the row do not differ statistically from each other by the Tukey test, with a significance of 5%. Means found in triplicate. Different lowercase letters on columns (a−c) indicate significant differences in thermal conditions ($p < 0.05$). Different capital letters on rows (A-C) indicate significant differences between formulations ($p < 0.05$)

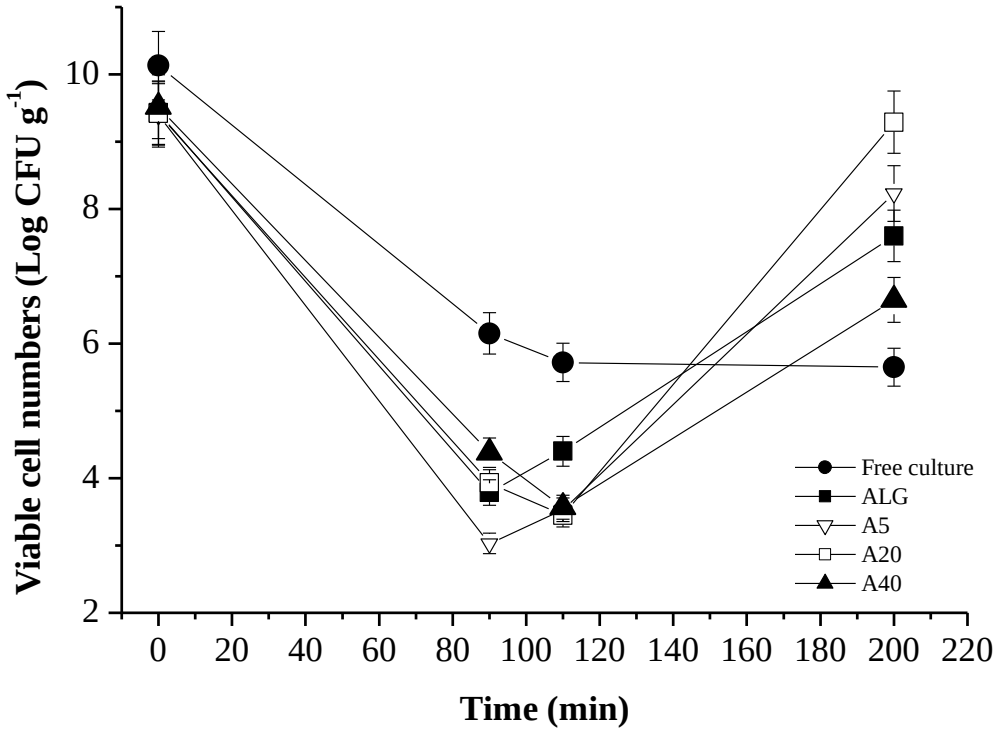

Fig. 2 - Survival of free and encapsulated *L. casei* in the different treatments after each stage of simulation of gastrointestinal conditions. ALG = 2% alginate particles; A5 = 2% alginate particles + 5% extract; A20 = 2% alginate particles + 20% extract; A40 = 2% alginate particles + 40% extract.

Table 4 – Effect of room temperature (25 ° C), refrigeration (7 ° C) and freezing (-18 ° C) on the viability of microparticles containing *L. casei* and red onion skin extract during storage for 90 days.

Room temperature (25 °C)					
Treatment Time (Days)	**Free culture**	**ALG**	**A5**	**A20**	**A40**
0	10.15 ± 0.02^{aA}	9.43 ± 0.07^{aBC}	9.39 ± 0.05^{aC}	9.42 ± 0.05^{aBC}	9.52 ± 0.01^{aB}
15	8.60 ± 0.09^{bB}	6.72 ± 0.07^{bD}	8.91 ± 0.05^{bA}	7.20 ± 0.05^{bC}	6.54 ± 0.19^{bD}
30	8.57 ± 0.05^{bA}	6.29 ± 0.02^{bD}	7.03 ± 0.00^{cB}	6.50 ± 0.00^{cC}	5.89 ± 0.04^{cE}
45	8.57 ± 0.01^{bA}	6.28 ± 0.05^{bC}	6.48 ± 0.04^{dB}	6.30 ± 0.03^{dC}	4.47 ± 0.03^{dD}
60	8.22 ± 0.05^{cA}	4.69 ± 0.27^{cC}	6.00 ± 0.01^{eB}	6.03 ± 0.04^{eB}	4.06 ± 0.05^{dD}
75	7.85 ± 0.01^{dA}	3.76 ± 0.56^{dC}	5.46 ± 0.12^{fB}	5.81 ± 0.05^{fB}	2.60 ± 0.30^{eD}
90	7.39 ± 0.09^{eA}	4.02 ± 0.06^{cdD}	5.24 ± 0.22^{fB}	4.86 ± 0.04^{gC}	1.80 ± 0.11^{fE}
Refrigeration temperature (7 °C)					
Treatment Time (Days)	**Free culture**	**ALG**	**A5**	**A20**	**A40**
0	10.15 ± 0.02^{aA}	9.43 ± 0.07^{aBC}	9.39 ± 0.05^{aC}	9.42 ± 0.05^{aBC}	9.52 ± 0.01^{aB}
15	6.55 ± 0.12^{bC}	9.35 ± 0.12^{aA}	9.45 ± 0.03^{aA}	9.39 ± 0.06^{aA}	7.27 ± 0.05^{bB}
30	5.83 ± 0.01^{cD}	8.10 ± 0.08^{bB}	7.46 ± 0.11^{bC}	8.42 ± 0.05^{bA}	3.75 ± 0.09^{cE}
45	5.09 ± 0.01^{dD}	7.68 ± 0.04^{bcA}	7.07 ± 0.07^{cB}	6.26 ± 0.10^{cC}	2.23 ± 0.11^{eE}
60	3.89 ± 0.02^{eC}	7.24 ± 0.31^{cdA}	7.32 ± 0.25^{bcA}	6.20 ± 0.15^{cB}	2.13 ± 0.04^{eD}
75	1.74 ± 0.23^{fE}	6.80 ± 0.09^{dB}	7.30 ± 0.04^{bcA}	5.90 ± 0.04^{dC}	3.04 ± 0.20^{dD}
90	1.46 ± 0.15^{fD}	6.31 ± 0.20^{eA}	6.67 ± 0.03^{dA}	5.56 ± 0.11^{eB}	3.26 ± 0.11^{dC}
Freezing temperature (-18 °C)					
Treatment Time (Days)	**Free culture**	**ALG**	**A5**	**A20**	**A40**
0	10.15 ± 0.02^{aA}	9.43 ± 0.07^{aBC}	9.39 ± 0.05^{aC}	9.42 ± 0.05^{aBC}	9.52 ± 0.01^{aB}
15	10.06 ± 0.07^{aA}	9.21 ± 0.02^{bB}	9.24 ± 0.01^{aB}	8.52 ± 0.04^{cC}	8.45 ± 0.18^{bC}
30	9.96 ± 0.00^{aA}	8.89 ± 0.10^{cC}	9.26 ± 0.03^{aB}	8.27 ± 0.01^{dD}	8.29 ± 0.03^{bD}
45	9.37 ± 0.04^{bAB}	8.55 ± 0.06^{dC}	9.27 ± 0.06^{aB}	9.48 ± 0.00^{aA}	7.84 ± 0.03^{cD}
60	9.08 ± 0.02^{bA}	8.25 ± 0.07^{eC}	8.67 ± 0.16^{bB}	8.91 ± 0.01^{bAB}	7.08 ± 0.18^{dD}
75	8.84 ± 0.53^{bA}	7.83 ± 0.01^{fB}	8.11 ± 0.03^{cB}	7.99 ± 0.08^{eB}	6.26 ± 0.10^{eC}
90	8.91 ± 0.04^{bA}	7.65 ± 0.11^{fB}	8.01 ± 0.06^{cB}	7.80 ± 0.03^{fB}	6.05 ± 0.28^{eC}

ALG = 2% alginate particles; A5 = particles of 2% alginate + 5% extract; A20 = particles of 2% alginate + 20% extract; A40 = particles of 2% alginate + 40% extract. Means followed by the same lowercase letter in the column and uppercase in the row do not differ statistically from each other by the Tukey test, with a significance of 5%. Means found in triplicate. Different lowercase letters on columns (a−c) indicate significant differences on the days ($p < 0.05$). Different capital letters on rows (A-C) indicate significant differences between formulations ($p < 0.05$)

5.4 ARTIGO III -

Bioactive strawberry pulp preparation by co-encapsulating *Lactobacillus casei* LC03 and red onion (*Allium cepa* L.) peel extract

Abstract

This study aimed to develop a functional strawberry pulp containing microencapsulated *Lactobacillus casei* and evaluate the effects of adding red onion peel extract at different concentrations (5, 20, and 40%) in the microparticles during storage and product consumption. The microparticle size and shape and encapsulation efficiency of the encapsulated bioactive compounds were evaluated. As for the pulp added from the microparticles, their physicochemical and microbiological features and probiotic survival under simulated gastrointestinal conditions and storage were analyzed; the size of the microparticles ranged from 136.00 and 305.00 µm. The encapsulation efficiency of the probiotics and compounds in the extract in the different treatments was satisfactory and varied from 77.77 to 92.11% for probiotic bacteria, 28.88 to 50.18% for reducing compounds, 35.72 to 69.01% for flavonoids, and 25.39 to 60.00% for total monomeric anthocyanins. The physicochemical and microbiological characteristics of the fruit pulp were within the required standards, regardless of the formulation evaluated. The formulations of alginate microparticles and alginate + 5% extract had the best results of *L. casei* probiotic viability in strawberry pulp under simulated gastrointestinal conditions and during storage at -18 °C for 60 days.

Keywords: microparticles, antioxidant**,** co-encapsulation, external gelation , probiotic.

1. Introduction

The search for a diet that transcends the need for nutrients and aims at awareness regarding environmental causes and balance with nature is pivotal, as veganism is no longer a trend, given it is growing exponentially and becoming a relevant market. Moreover, to achieve a better quality of life, the search for healthy and bioactive foods has also become part of the dietary pattern of the vegan and general population.

According to Kleerebezem et al. (2019), probiotic foods are widespread and consumed by millions of people every day. Nonetheless, most of the probiotic products available today are of dairy origin (Champagne, Gardner, & Roy, 2005); therefore, there is an undeniable need to expand the development of probiotic products and multiply the number of alternatives to meet the demands of consumer groups.

As part of a healthy diet, it is recommended to consume 400 g or five servings of fruits and vegetables every day (WHO, 2003; Mondini, Moraes, Freitas, & Gimeno, 2010). Fruits have numerous vitamins, fiber, minerals, and polyphenols (Harborne & Williams, 2000; Jaime, Figueiredo, Moura, & Malta, 2009), which decrease the incidence of diseases such as cancer and cardiovascular diseases (Williamson, 1996). Thus, developing new fruit-based probiotic products is a healthy alternative with high acceptability for all audiences, including vegans, vegetarians, and people who are intolerant or allergic to milk.

Strawberries are capable of exerting antioxidant, diuretic, and anti-inflammatory effects; these fruits improve muscle development and regeneration and exert positive effects on the skin, teeth, and bones, and act in collagen formation in the body (Rocha, Abreu, Corrêa, Santos, & Fonseca, 2008; Andrade, Diniz, Neves, & Nóbrega, 2002; Nunes & Novello, 2020). However, strawberries have low perishability due to their high post-harvest metabolic rates and must be consumed within a week (Alves, Alencar, Ferreira, Silva, & Ribeiro, 2019). With this in mind, the use of fruit pulps reduces losses and enables all harvest fruits to be fully taken advantage of (Dantas et al., 2012). The fruit pulp is defined as the unfermented product, not concentrated, not diluted, and obtained from pulpy fruit through the appropriate technological process, with a minimum content of total solids from the edible part of the fruit (Brazil, 2000); the most common way to preserve fruit pulp nowadays is by freezing (Carvalho, Mattietto, & Beckman, 2017).

Given that it presents low pH, high acidity, and antimicrobial compounds, inserting probiotic bacteria into strawberry pulp is challenging because these microorganisms are highly sensitive. In order to form a protective barrier that prevents probiotics from interacting

with food, microencapsulation presents itself as a highly effective alternative. Associated with this, the use of bioactive extracts in the process of forming microparticles may prove effective in increasing probiotic survival.

Numerous studies have already reported quercetin and its ability to modulate intestinal microflora (Etxeberria et al., 2015; Porras et al., 2017). Onions stand out as one of the vegetables with the highest levels of flavonoids, especially quercetin (Santiago et al., 2020; Campone et al., 2018), and higher quantities of this compound are found in the peel than in any other part of the vegetable (Shim, Yi, & Kim, 2011), in addition to reducing compounds and anthocyanins, which, similarly to quercetin, have high antioxidant properties. The use of onion peel is also an alternative to the increasing demand for food worldwide and generates a tremendous amount of waste, which often has high aggregate potential (Mekar Saptarini & Herawati, 2018).

Given this scenario, this study aimed to evaluate the influence of a red onion (*Allium cepa* L.) peel extract at different concentrations (5, 20, and 40%) on probiotic microparticle viability added to frozen strawberry pulp.

2. Materials and Methods

The red onions were purchased locally in Santa Maria (Rio Grande do Sul State, southern Brazil). The onion peels were manually removed and sanitized with hypochlorite; the peels were then dried in an oven at 50 °C (± 1) for 24 h, ground in a knife mill (Mill MA 630/1-Marconi; 5 rpm for 10 s), and stored in a freezer at -18 °C until analyses.

The probiotics of the species *Lactobacillus casei* LC03 were kindly provided by the company Coana (Florianópolis, Brazil). For culture activation, 1 g was incubated in 100 mL of MRS broth (Himedia) for 15 to 18 h at 37 ± 1 °C. It was then centrifuged at 4670 × g for 15 min at 4 ± 1 °C and washed with saline (0.85%).

2.1 Obtaining the red onion peel extract

Hydroalcoholic extracts were prepared from 3 g of ground red onion peel and then mixed with 60 mL of 80% ethanol at 25 °C under stirring; the extraction time was 120 min according to Viera et al. (2017). Afterward, ethanol was evaporated from the extract using a rotary evaporator, and its volume was corrected with distilled water.

2.2 Reducing capacity

The reducing capacity was evaluated using the Folin-Ciocalteu method according to Roesler et al. (2007). The absorbances obtained in the reaction were read in a spectrophotometer at a wavelength of 765 nm, and the content of reducing compounds was expressed as milligrams of gallic acid/g of dry sample (mg GAE g^{-1})

2.3 Total flavonoid content

The total flavonoid content was evaluated according to Zhishen, Mengcheng, & Jianming (1999). The absorbances obtained in the reaction were read in a spectrophotometer at a wavelength of 510 nm, and the flavonoid content was expressed as mg quercetin (QE) equivalent per g of dry sample (mg QE/g).

2.4 Total monomeric anthocyanin content

The total monomeric anthocyanin content was performed according to Giusti & Wrolstad (2001) using the differential pH method. The absorbances obtained in the reaction were read at the wavelengths of 520 and 700 nm, and the total monomeric anthocyanin content was calculated using the following equation:

Anthocyanin content (mg/100 g dry matter) = A x MW x DF⁄ (ε x W)

Where A is the absorbance (520 – 700 nm) pH 1.0 - (520 – 700 nm) pH 4.5, MW is the molecular weight of cyanidin-3glucoside ($C_{15}H_{11}O_6$, 449.2), DF is the dilution factor, ε is the molar absorptivity (26900), and W is the sample weight (g).

2.5 Antioxidant capacity

The oxygen radical absorbance capacity (ORAC) was analyzed as proposed by Ou et al. (2001), and the results were expressed as μmol equivalents of Trolox per gram of red onion peel (μmol of Trolox/g).

2.6 Microparticle production by external ionic gelation

The microparticles were produced using the method described by Etchepare et al. (2016), with adaptations. For this, 100 mL of a solution containing 2% alginate was prepared along with the activated probiotic *L. casei* LC03 and red onion peel extract. A dual fluid atomizer nozzle (0.3 mm) at 0.125 kgf/cm was used, and this mixture was sprayed in 0.1 M $CaCl_2$, where it remained under stirring for 30 min. From this method, four microparticle

formulations were obtained: 2% alginate microparticles (AM), 2% alginate microparticles + 5% extract (AME5), 2% alginate microparticles + 20% extract (AME20), and 2% alginate microparticles + 40% extract (AME40).

2.7 Microparticle characterization

2.7.1 Optical microscopy and particle size

Optical microscopy was performed using an optical microscope (Carl Zeiss Axio Scope A1, Oberkochen, Germany) equipped with an Axio Cam MRc digital camera (Carl Zeiss). The size of the microparticles was measured by laser diffraction using the Mastersizer 2000 particle size analyzer (Malvern, UK).

2.7.2 Encapsulation efficiency of reducing compounds, flavonoids, and total monomeric anthocyanin content

The extraction of reducing compounds, flavonoids, and total monomeric anthocyanin content from inside the microparticles was performed according to Robert et al. (2010), with adaptations. To this end, 0.5 mL of acetonitrile and 0.5 mL of methanol:acetic acid:water (50:8:42 mL/mL/mL) and 1 g of microparticles were vortexed for 4 min and then sonicated for 60 min. Afterward, the supernatant was collected and centrifuged at 5000 rpm for 15 min. From this liquid, the encapsulation efficiency of reducing compounds, flavonoids, and total monomeric anthocyanin content were determined as described in sections 2.2, 2.3, and 2.4, respectively, by calculating the percentage of reducing compounds, flavonoids, and total monomeric anthocyanin content encapsulated over the total of these compounds in the initial alginate solution.

2.7.3 Probiotic encapsulation efficiency

The probiotic encapsulation efficiency (EE%) was calculated as proposed by Martin, Lara-Villoslada, Ruiz, & Morales (2013) in Eq. 1.

$$\text{Eq. 1. } EE = (N/N0) \times 100$$

Where N is the number of viable cells (log CFU g^{-1}) released by breaking down the microparticles and N0 is the number of viable cells (log CFU g^{-1}) in the cell concentrate before microencapsulation.

2.8 Preparation of the frozen strawberry pulp with added microparticles containing *L. casei* and red onion (*Allium cepa* L.) peel extract

Organic strawberries were purchased in Agudo (Rio Grande do Sul State), sanitized with running water, processed in a blender, and sieved to prepare the pulp. Then, 5% microparticles (5 g/100 g) were added to the pulp and homogenized. The pulp was then packed in polyethylene bags and stored at -18 °C for further analysis.

2.9 Microbiological analysis in the frozen strawberry pulp

Microbiological analyses to detect *Salmonella* sp., total coliforms, thermotolerant coliforms, and molds and yeasts were performed according to Brazil (2001). Probiotic quantification was performed according to Sheu, Marshall, & Heymann (1993), with modifications. The probiotics were released from the microparticles using a sterile phosphate buffer solution (pH 7.5; 25 g/225 mL). Serial decimal dilutions were then performed in 0.1% peptone water with plating of 1.0 mL of the dilutions in triplicate onto sterile Petri dishes, followed by adding MRS Agar. The plates were then incubated at 37 ± 1 °C for 72 h under anaerobic conditions.

2.10 Physicochemical analysis in the frozen strawberry pulp

For the physicochemical characterization of the pulp, the pH, soluble solids, moisture, and titratable acidity were evaluated according to the method of the Adolfo Lutz Institute (IAL, 2009).

2.11 Resistance of free and microencapsulated probiotics in strawberry pulp under simulated gastrointestinal conditions

Simulation of esophagus/stomach (pH 2.0/90 min in the presence of pepsin), duodenum (pH 5.0/20 min in the presence of pancreatin and bile salts), and ileum (pH 7.5/90 min) conditions were performed according to the method described by Madureira, Amorim, Gomes, Pintado, & Malcata (2011) with adaptations. The analysis was performed in a TE 421 shaker (Tecnal, Piracicaba, São Paulo, Brazil) at 37 ± 2 °C to simulate body temperature. At the end of each simulation stage, aliquots were collected and plated according to item 2.9.

2.12 Evaluation of the shelf life of free and microencapsulated probiotics in the strawberry pulp during storage and under freezing conditions

The probiotic viability of the strawberry pulp was evaluated for 60 days at -18 ± 1 °C to simulate freezing conditions. Counts were performed every 15 days according to item 2.9.

2.13 Statistical analyses

Data were submitted to analysis of variance (ANOVA) using the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA), followed by Tukey's test for comparison of means, considering a significance level of 5% ($p<0.05$). Tests were performed in triplicate, and data were expressed as mean ± standard deviation.

3. Results and discussion

3.1 Optical microscopy and microparticle size

Based on the morphological analyses of the microparticles (Figure 1), all treatments showed spherical or elliptical shapes and an intact structure without visible cracks or fissures. The advantages of obtaining spherical-shaped microparticles include easier consumption, manufacturing, and packaging (Nami, Lornezhad, Kiani, Abdullah, & Haghshenas, 2020).

Furthermore, we observed that adding the red onion peel extract altered the color of the microparticles, as shown in Figures A, B, C, and D, which correspond to treatments AM, AME5, AME20, and AME40, respectively. The higher the extract concentration added to the microparticles, the darker the coloration. This coloration, which can vary from red to purple, is due to anthocyanins in the red onion peel extract; anthocyanins are natural pigments characterized as phenolic compounds of the flavonoid family and confer coloration to a plethora of flowers, fruits, and plants (Chopra & Panesar, 2010; Nascimento et al., 2008). Moreover, incorporating anthocyanins in foods grants them bioactive and medicinal properties given their high antioxidant activity (Maran, Priya, & Manikandan, 2014).

As for the size of the microparticles, there was a variation from 136.00 to 305.00 ± µm, with the smallest size being obtained in the AM treatment and the largest size in the AME20 treatment (Table 1). Treatments AME5 and AME40 showed 214.00 and 205.00 µm sizes, respectively. The sizes obtained in this study are advantageous because, in the micrometer range, the microparticles allow the food to remain smooth, while in the millimeter range, the particles confer a sandy texture to the product (Mohammad Ali Khosravi Zanjani, 2012)

Nami, Lornezhad, Kiani, Abdullah, & Haghshenas (2020) employed an extrusion technique and reported even larger particles (860-1130 µm) using 2% alginate. Nevertheless,

the authors did not observe any adverse effect on the texture and structure of orange juice mixed with these particles. Similarly, Batista de Oliveira et al. (2021) maintained the average acceptance for the consistency of chocolate milk with microencapsulated *Spirulina sp*. LEB 18 and reported microparticles that varied from 616.60 to 680.75 μm.

It was also possible to conclude that the composition of the wall material interferes with the size of the microparticles, as shown in Table 1. The addition of the red onion peel extract at different concentrations (5, 20, and 40%) significantly increased the size of the microparticles compared to the treatment that only contained alginate (AM).

3.2 Probiotic encapsulation efficiency, reducing compounds, flavonoids, and total monomeric anthocyanin content

Encapsulation efficiency (EE%) above 77 % indicates the successful entrapment of the probiotic *L. casei* inside the microparticles prepared in the different formulations (AM, AME5, AME20, and AME40; Table 1). These high EE% values are pivotal so that when they are inserted into food, the microparticles contain a sufficiently high number of probiotics that survive the processing and storage conditions of the product and reach their site of action in adequate amounts. Microencapsulation, in general, is one of the best ways to protect sensitive bioactive compounds; in external ionic gelation, other authors have reported EE% >90% (Angélica Andrade Lopes et al., 2020), as well as in other microencapsulation techniques, including complex coacervation with EE% >90.9% (da Silva et al., 2019), internal emulsification/ionic gelation with EE% >66.2% (Raddatz et al., 2020), and spray drying with EE% = 94.61% (Rosolen et al., 2019).

Among the different microparticle formulations, AME40 showed the highest EE% compared to the others, reaching a value above 92%. As for the compounds present in the extract, before microencapsulation, the content of reducing compounds was 653.75 ± 32.78 mg/g, the flavonoid content was 78.49 ± 0.34 mg/g, the total monomeric anthocyanin content was 47.09 ± 7.65 mg/g, and the antioxidant capacity was 1096.45 μmol Trolox/g (data not tabulated). Onions contain the highest amount of flavonoids and organosulfur compounds (Pareek, Sagar, Sharma, & Kumar, 2017). Viera et al. (2017) obtained values of 640.8, 35.2, and 30.9 mg/g of total reducing compounds, flavonoids, and monomeric anthocyanin content, respectively, when producing red onion peel extracts using this extraction method.

As for the EE% of the reducing compounds, the AME20 treatment stood out, with 50.18 ± 0.01%. Arriola et al.(2018) obtained EE% of total phenolic compounds above 60% when using the extrusion/external ionic gelation technique to encapsulate stevia extract. The

use of alginate has also already demonstrated excellent EE% results when encapsulating anthocyanins (~89%) and phenolic compounds (~98%) extracted from jabuticaba fruit (*Plinia cauliflora* (Mart.) Kausel) and Tubuna (*Scaptotrigona bipunctata*) stingless bee propolis peels by ionotropic gelation (Dallabona et al., 2020).

As for the EE% of flavonoids, AME5 obtained the highest values (69.01 ± 0.01%); this result is similar to the data reported by Milea et al. (2019), who achieved 66.46 ± 0.18% as the best result of EE% of flavonoids from yellow onion peel, although the authors employed freeze-drying. Lastly, the EE% of total monomeric anthocyanin content was higher in the AME20 and AME40 treatments with values of 60.00 ± 0.08 and 59.95 ± 0.05, respectively. Enache et al. (2020) obtained EE% of anthocyanins from blackcurrant fruits of 95.46 ± 1.30% and 87.38 ± 0.48% from the probiotic *Lactobacillus casei* when encapsulating them into a single matrix, albeit the authors used microencapsulation via freeze-drying.

3.3 Physicochemical and microbiological characteristics of fruit pulp containing microencapsulated *L. casei* in the different treatments

The physicochemical evaluations in the strawberry pulps containing the free or microencapsulated *L. casei* probiotic are listed in Table 2. According to the general technical regulation to set the identity and quality standards for fruit pulp set by legislation (Brazil, 2001), the strawberry pulp is the product obtained from the edible part of the strawberry (*Fragaria x. ananassa* Duchesne, *Fragaria chiloensis Duchesne* x *Fragaria virginiana* Duchesne) through an appropriate technological process.

The regulation establishes the minimum values of 3.3 for pH, 6.5 for soluble solids in °Brix at 20 °C, and 0.8 for titratable acidity (g/100 g). All the samples evaluated were above these minimum values required. In addition, the moisture content of the different samples was evaluated and ranged from 90.69 to 91.12%, with no statistical differences among the five treatments. Fruits have high moisture content with large variations among them. In addition, the water supply to the plant and growing conditions interfere with the moisture content of fruit (Chitarra & Chitarra, 2005).

The results of the microbiological evaluations of pathogenic or spoilage bacteria in strawberry pulp containing free or microencapsulated *L. casei* probiotic are described in Table 3. The analyses were based on the standards required by RDC No. 12 (Brazil, 2001), which establishes the microbiological standards for foods in Brazil.

According to the results presented herein, the strawberry pulps analyzed are suitable for consumption, since no salmonella, fecal coliforms, and total coliforms were observed in

all samples evaluated (Table 3), with these results being in agreement with the legislation, which has a maximum tolerable limit of 10^2 NMP/g of fecal coliforms and absence of *Salmonella* spp. in 25 g of sample. The thermotolerant coliforms may signal contamination of fecal origin or by other enteric microorganisms, which are associated with risks to human health (Verma, Saharan, Nimesh, & Singh, 2018). *Salmonella sp.* is one of the main microorganisms that cause food outbreaks worldwide, causing economic losses due to its high morbidity (Sharma & Carlson, 2000).

The National Sanitary Surveillance Agency (ANVISA) does not determine limits for molds and yeasts in fruit pulps; nonetheless, these microorganisms were evaluated based on Normative Instruction No. 4, which establishes maximum counts of $5x10^3$/g for *in natura* pulp, frozen or not (Brazil, 2018). All samples evaluated were in compliance with current legislation, with counts of $1.06 \times 10^2 \pm 5.77$, $1.56 \times 10^2 \pm 23.09$, $4.50 \times 10^2 \pm 36.30$, $8.40 \times 10^2 \pm 22.81$, $8.93 \times 10^2 \pm 13.85$ for free culture, AM, AME5, AME20, and AME40, respectively. High mold and yeast counts represent a potential spoilage capacity as well as a public health risk, considering that some filamentous fungi are toxin-producing organisms (Moraes & Machado, 2021).

Therefore, the data obtained in the microbiological evaluations indicate that the strawberry pulps were prepared under adequate hygienic and sanitary conditions and did not represent a threat to the consumers.

3.4 Survival of free and microencapsulated *L. casei* in fruit pulp after simulating gastrointestinal conditions.

The survival of the free or microencapsulated *L. casei* probiotic in strawberry pulp under simulated gastrointestinal conditions is listed in Table 4. Stress conditions such as digestive enzymes and low pH have negative and inhibitory effects on probiotic performance and viability. Overcoming these challenges is paramount for probiotics to reach the ileum (their site of action) and colonize it to promote beneficial effects to the host organism (Zeashan et al., 2020). As shown in Table 4, the strawberry pulp containing the free culture lost its viability in the first stage of the simulated gastrointestinal conditions, reaching the esophagus/stomach with only 3.66 ± 0.03 log CFU/g^{-1}. This result demonstrates the need for a system that ensures that the probiotic *L. casei* reaches the ileum without remaining in direct contact with gastric fluids or the product in which it is inserted.

Furthermore, we observed that the microencapsulation system used in this study met such a need, with the strawberry pulps containing the microparticles from treatments AM and

AME5 releasing the probiotic cells in sufficient quantities and at the appropriate time (6.51 ± 0.48 and 6.73 ± 0.23 log CFU/g^{-1}, respectively). Alginate is extremely efficient in releasing trapped compounds at neutral pH, such as intestinal pH (Annan, Borza, & Hansen, 2008; Gul & Dervisoglu, 2017). Higher probiotic bacteria viability was also reported by Chandramouli, Kailasapathy, Peiris, & Jones (2004) at pH 2.0 when alginate was used for encapsulation.

Other microencapsulation methods, such as freeze-drying, are also efficient in protecting *L. casei* probiotics from simulated gastrointestinal conditions, with increased survival of the encapsulated probiotic, while there was a loss of viability of the free probiotic (Li et al., 2019). The strawberry pulps that contained the probiotic microparticles together with the highest concentrations of red onion skin extract (AME20 and AME40) did not reach the ileum in the necessary amounts (6 log CFU/g).

Reza Gheisari, Davar, & Shahram Shekarforoush (2018) also evaluated microencapsulation process efficiency on the probiotic *L. casei* under simulated gastrointestinal conditions when added to mango juice. The microencapsulation was performed by extrusion with calcium alginate and chitosan, and similarly to our study, the authors observed higher probiotic survival than the free microorganism. The same occurred in the study of Marcial-Coba, Saaby, Knøchel, & Nielsen (2019) for *Akkermansia muciniphila* and *L. casei* added in chocolate in free form or microencapsulated by extrusion followed by lyophilization after gastric passage *in vitro*, with higher survival of microencapsulated strains.

3.5 Viability of free and microencapsulated *L. casei* added to strawberry pulp stored at freezing temperatures

The survival of the free and microencapsulated *L. casei* LC03 probiotic in the strawberry pulp during storage at -18°C was evaluated, and the results are presented in Figure 2. To develop new probiotic foods, an important factor is to verify how they adapt to the food matrix as probiotics must be consumed regularly, and in order to obtain their health benefits, their presence in foods must be in sufficient quantities (at least 10^6 CFU/g or mL^{-1}) during the shelf life (Abadía-García et al., 2013).

Probiotic bacteria are recommended to be inserted into food formulations with relatively high pH, around 5.5 to 6.5 (Cruz, Antunes, Sousa, Faria, & Saad, 2009). In addition, freezing conditions may cause cellular damage to these microorganisms, leading to a reduction of up to 1 log CFU/g (Cruz et al., 2011). It was possible to observe that the free culture was not resistant to the acidity, low pH, and freezing conditions of the strawberry pulp since, in less than 15 days, its counts were already below the required standard (6 log CFU/g^{-}

[1]) for the product to be considered a probiotic (Figure 2). Despite the low pH of the strawberry pulp developed in this study (3.48-3.85), we noted that the microencapsulation allowed the survival of the microorganism *L. casei* LC03, highlighting the AM treatment, which kept the probiotics viable until the end of 60 days (6.92 log CFU/g), followed by the AME5 treatment, with viability between 45 and 60 days. The AME20 treatment lost viability after 30 days of storage, while AME40 remained viable for less than 15 days.

In addition to this study, Afzaal et al. (2020) also reported significant improvements in the viability of microencapsulated *Lactobacillus casei* added to ice cream for 80 days at -20 °C, in addition to higher resistance under simulated gastrointestinal tract conditions compared to the free microorganism in the same product. In another study, microencapsulation also proved effective in protecting the probiotic *Lactobacillus casei* ATCC 334, since, when added to cashew ice cream at -18 °C for 150 days, the probiotic microencapsulated by extrusion with sodium alginate and chitosan showed higher survival than the free microorganism (Farias, 2017).

As for the concentration of red onion (*Allium cepa* L.) peel extract in the microparticles, we observed that the higher the extract concentration, the greater the loss of probiotic viability over the 60 days. Thus, the lowest concentration of extract (5%) proved to be the most effective in maintaining the shelf life of the probiotic *L. casei* LC03. The bioactive compounds present in onions have, among their various properties, antibacterial action (Griffiths, Trueman, Crowther, Thomas, & Smith, 2002), which may explain the fact that the highest concentrations of extract used in this study (20 and 40%) resulted in unfavorable effects on the viability of the probiotic *L. casei* LC03. The way plant extracts act is not yet completely defined; according to several studies, the compounds in the extracts may lead to the permeabilization or rupture of the cytoplasmic membrane of microorganisms, exposing the contents of the cytoplasm, besides being able to produce inhibitory effects on the ATPase enzyme and, consequently, causing cell death (Gill & Holley, 2006; Zhang et al., 2016).

Sagdic, Ozturk, Cankurt, & Tornuk (2011) supplemented ice cream containing *L. casei* Shirota with ellagic acid, gallic acid, grape seed extract, pomegranate peel extract, and peppermint essential oil and investigated the interaction of the probiotics with these compounds after 60 days at -18 °C. The phenolic compounds were found to benefit the survival of the probiotics.

Lastly, as described herein, it was possible to obtain a polyfunctional product by successfully applying the probiotic *L. casei* with a red onion peel extract to a highly nutritious food matrix that numerous vitamins, fibers, minerals, and polyphenols.

4. Conclusions

The red onion peel extract can be efficiently used to microencapsulate the probiotic *L. casei* LC03 to ensure viability and stability in new functional foods, such as the strawberry pulp. It was possible to observe that low extract concentrations are enough to achieve a protective and functional effect in the microcapsule, with probiotic viability under simulated gastrointestinal conditions and storage at -18 °C with only 5% extract. Therefore, this study allowed the development of a new probiotic product capable of satisfying the most diverse publics, including vegetarians, vegans, and those intolerant to gluten and lactose.

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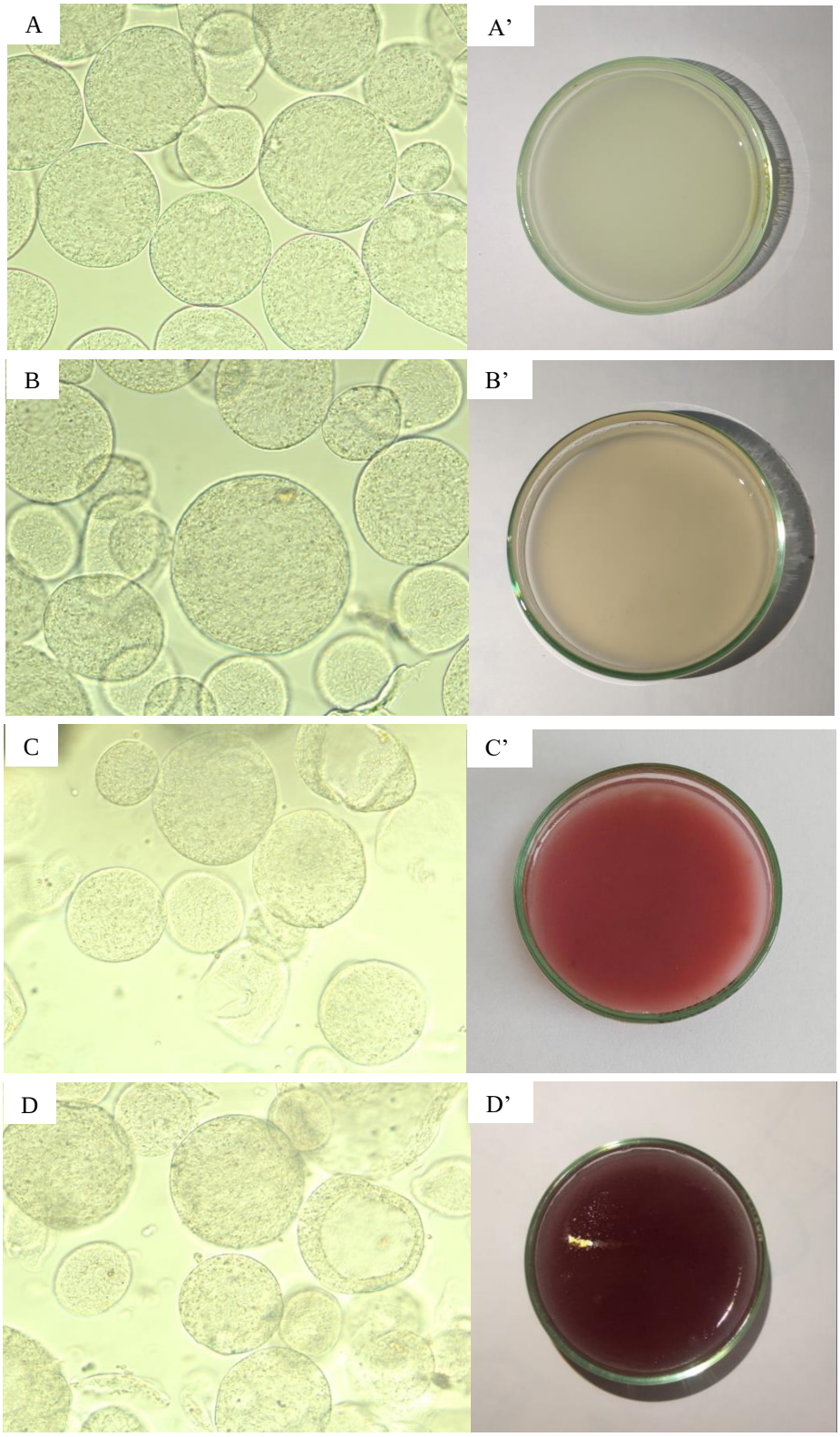

Fig 1. Optical microscopy of alginate microparticles with different concentrations of red onion peel extract containing *L. casei* obtained by external ionic gelation. A = 2% alginate microparticles (AM) (40x); B = 2% alginate microparticles + 5% extract (AME5) (40x); C = 2% alginate microparticles + 20% extract (AME20) (40x); D = 2% alginate microparticles + 40% extract (AME40) (40x).

Table 1 – Characteristics of particles containing *L. casei* obtained by external ionic gelation with alginate and red onion peel extract at different concentrations.

Treatment	Particle size (µm)	EE% probiotics	EE% reducing compounds	EE% flavonoids	EE% total monomeric anthocyanins
AM	136.00	80.30 ± 1.42^{b}	-----	-----	------
AME5	214.00	77.77 ± 0.43^{c}	30.10 ± 0.06^{b}	69.01 ± 0.01^{a}	25.39 ± 0.05^{b}
AME20	305.00	79.35 ± 0.92^{bc}	50.18 ± 0.01^{a}	35.72 ± 0.00^{c}	60.00 ± 0.08^{a}
AME40	205.00	92.11 ± 0.23^{a}	28.88 ± 0.01^{b}	41.28 ± 0.00^{b}	59.95 ± 0.05^{a}

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract. EE%= percentage of encapsulation efficiency. The means followed by the same lowercase letter in the column do not differ statistically from each other by Tukey's test, with a significance of 5%. Means found in triplicate.

Table 2 - Physicochemical characterization of whole strawberry pulp with or without alginate microparticles with different concentrations of red onion peel extract containing *L. casei* obtained by external ionic gelation.

Treatment	pH	Soluble Solids / Brix	Moisture	Titratable Acidity
Free culture	3.61 ± 0.01^{bc}	7.80 ± 0.17^{a}	91.12 ± 0.13^{a}	1.03 ± 0.05^{a}
AM	3.62 ± 0.01^{bc}	7.40 ± 0.17^{ab}	90.69 ± 0.68^{a}	0.86 ± 0.05^{a}
AME5	3.85 ± 0.12^{a}	7.50 ± 0.20^{ab}	91.09 ± 0.09^{a}	1.0 ± 0.00^{a}
AME20	3.48 ± 0.00^{c}	7.33 ± 0.10^{b}	90.75 ± 0.51^{a}	0.86 ± 0.05^{a}
AME40	3.70 ± 0.02^{ab}	7.43 ± 0.15^{ab}	90.73 ± 0.28^{a}	1.0 ± 0.1^{a}

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract.

Free culture = strawberry pulp containing free *L. casei.*

The means followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test, with a significance of 5%. Means found in triplicate.

Table 3 – Microbiological characteristics of strawberry pulp added with microparticles containing *L. casei* obtained by external ionic gelation with alginate and red onion peel extract at different concentrations.

Treatment	Molds and Yeasts	Total Coliforms	Thermotolerant Coliforms	Salmonella
Free culture	$1.06 \times 10^2 \pm 5.77$	Absent	Absent	Absent
AM	$1.56 \times 10^2 \pm 23.09$	Absent	Absent	Absent
AME5	$4.50 \times 10^2 \pm 36.30$	Absent	Absent	Absent
AME20	$8.40 \times 10^2 \pm 22.81$	Absent	Absent	Absent
AME40	$8.93 \times 10^2 \pm 13.85$	Absent	Absent	Absent

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract. The means followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test, with a significance of 5%. Means found in triplicate.

TabLE 4 – Survival of free and encapsulated *L. casei* in different treatments after each stage of the simulation of gastrointestinal conditions.

	Free culture	**AM**	**AME5**	**AME20**	**AME40**
Initial count	9.14 ± 0.06^{aB}	8.45 ± 0.15^{aC}	8.19 ± 0.04^{aD}	8.35 ± 0.09^{aCD}	9.70 ± 0.02^{aA}
Esophagus / stomach 90 min / pH 2.0	$3,66\pm0.03^{bB}$	4.66 ± 0.07^{cA}	4.28 ± 0.07^{cA}	4.72 ± 0.04^{cA}	4.31 ± 0.36^{cA}
Duodenum 20 min / pH 5.0	$3,58\pm0.05^{bD}$	4.51 ± 0.03^{cB}	4.17 ± 0.09^{cC}	4.43 ± 0.12^{dB}	5.31 ± 0.00^{bA}
Ileum 90 min / pH 6.5	$2,91\pm0.31^{cD}$	6.51 ± 0.48^{bA}	6.73 ± 0.23^{bA}	5.22 ± 0.10^{bB}	3.71 ± 0.02^{dC}

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract.

Free culture = strawberry pulp containing free *L. casei.*

The means followed by the same lowercase letter in the column and uppercase in the row do not differ statistically from each other by the Tukey test, with significance set at 5%. Means found in triplicate.

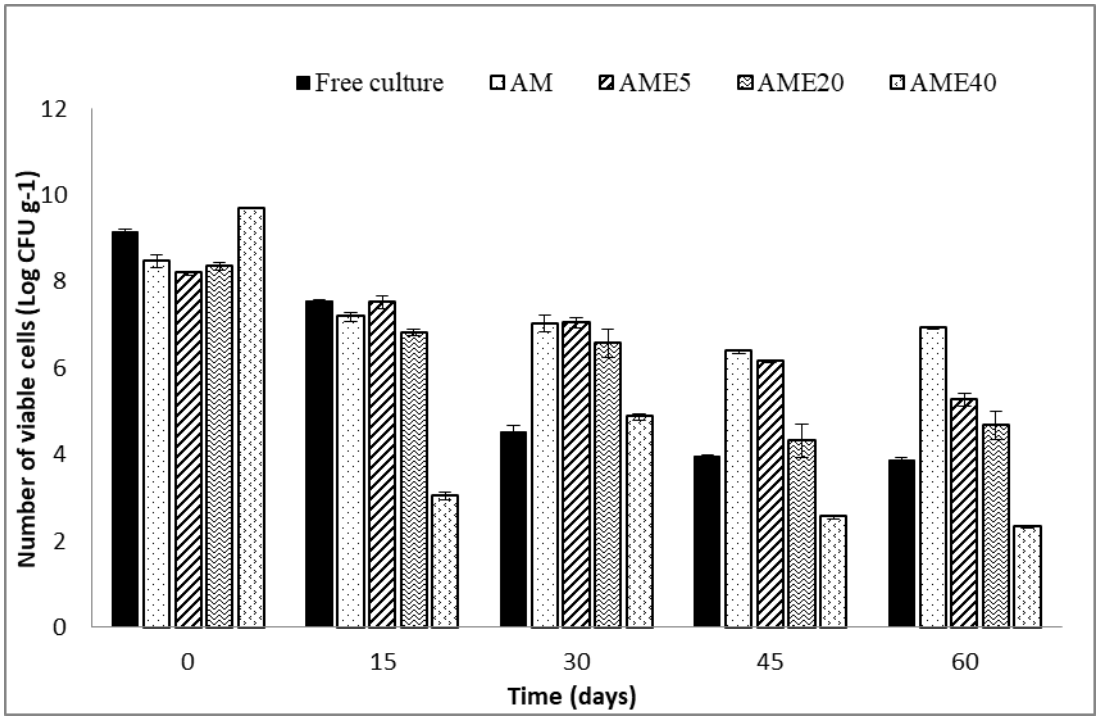

Figure 2 – Effect of storage under freezing temperature (-18 °C) on the viability of free and microencapsulated *L. casei* added to strawberry pulp..

AM = 2% alginate particles; AME5 = particles of 2% alginate + 5% extract; AME20 = particles of 2% alginate + 20% extract; AME40 = particles of 2% alginate + 40% extract.

6. DISCUSSÃO GERAL

6.1 CARACTERIZAÇÃO DAS MICROPARTÍCULAS

6.1.1 Morfologia e tamanho das micropartículas

A partir da observação por microscópio óptico, de modo geral as micropartículas dos Artigos II e III apresentaram estrutura uniforme, com formato esférico ou elíptico, o que é característico da técnica de gelificação iônica externa e já foi relatado por diversos outros autores (ALKHATIB, 2020; DE CÁSSIA SOUSA MENDES et al., 2021).

A análise de tamanho das micropartículas apresentou resultados dessemelhantes entre os dois manuscritos, visto que no Artigo II, o tamanho variou de 149.29 a 167.05 μm e no Artigo III a variação foi de 136.00 a 305.00 μm.

Uma micropartícula pode possuir uma faixa de tamanho variando entre 0,2 μm a 5 mm (SILVA et al., 2014). ARRIOLA et al. (2019) relataram modificações estruturais e no aspecto de cápsulas de alginato quando incorporadas de extrato aquoso de Stevia Rebaudiana Bertoni. Uma possível explicação para a variação de tamanho das micropartículas nesse estudo, é que o extrato de casca de cebola roxa afetou as ligações dos íons cálcio com os grupos carboxila do alginato.

Ressalta-se que o melhor tamanho para uma micropartícula está diretamente ligado à matriz na qual será inserida, devendo ser pequena o bastante para que não seja perceptível sensorialmente no alimento quando ingerido.

6.1.2 Eficiência de ecapsulação

Ao se avaliar a eficiência de encapsulação probiótica, em ambos os artigos (II e III) houve o aprisionamento em quantidades satisfatórias do microrganismo *L.casei* LC03, com resultados variando de 92.28 ± 0.08 a 93.95 ± 0.15 % no Artigo II e de 77.77 ± 0.43 a até 92.11 ± 0.23 % no Artigo III. Estes resultados são semelhantes aos de PUPA et al. (2021) que encapsularam diferentes probióticos por extrusão utilizando alginato e quitosana a partir da metodologia de extrusão e alcançaram EE% de até 93.52%.

Para verificar a eficiência de aprisionamento do extrato de casca de cebola roxa nas micropartículas, o teor de compostos redutores, flavonoides e antocianinas monoméricas totais foi caracterizado e comparado ao conteúdo destes compostos nas diferentes formulações

para produção de micropartículas (com 5, 20 e 40% de extrato). No Artigo II a maior retenção de compostos redutores se deu no tratamento A5, já para flavonoides e antocianinas monoméricas totais, a retenção foi maior no tratamento A20. Em contrapartida, no Artigo III, as maiores retenções foram no tratamento AME20 para compostos redutores, tratamento AME5 para flavonoides e tratamentos AME20 e AME40 para antocianinas monoméricas totais.

Apesar dessa discrepância entre os dois Artigos, os diferentes compostos bioativos se mostraram presentes no interior de todas as micropartículas, podendo auxiliar na sobrevivência do probiótico durante a sua encapsulação, processamento, armazenamento e consumo. A técnica de gelificação iônica externa também já se mostrou eficaz para o aprisionamento de extratos bioativos em outros estudos (DE CÁSSIA SOUSA MENDES et al. 2021; PASUKAMONSET,KWON; ADISAKWATTANA, 2016; KO, KOO; PARK, 2008).

6.2 VIABILIDADE DE *Lactobacillus casei* LC03 APÓS EXPOSIÇÃO A CONDIÇÕES GASTROINTESTINAIS SIMULADAS

Garantir que os probióticos sobrevivam ao baixo pH do estômago quando ingeridos está dentre os maiores motivos para se encapsular esses microrganismos (HEIDEBACH; FÖRST; KULOZIK, 2009).

Ao se observar o probiótico não encapsulado após a simulação gastrointestinal, em ambos os artigos (II e III), sua viabilidade foi comprometida, pois ele não atingiu o íleo em quantidades necessárias (acima de 6 log), o que torna evidente a sensibilidade desses microrganismos a meios de baixo pH e com presença de enzimas digestivas.

Ao contrário disso, ao se observar os probióticos microencapsulados nos tratamentos ALG, A5, A20 e A40 do Artigo II quando submetidos a essas mesmas condições, sua viabilidade foi mantida, com todos os tratamentos atingindo o íleo em quantidades acima do recomendado, com destaque para o tratamento A20, que apresentou maiores contagens em relação aos demais.

Da mesma forma, no Artigo III, também foi possível manter as contagens probióticas acima de 6 log ao final da simulação gastrointestinal nos tratamentos AM e AME5, graças ao processo de microencapsulação. A microencapsulação também já se mostrou necessária em outros estudos para garantir a viabilidade de probióticos frente a fluidos gástricos simulados (DÍAZ-VERGARA et al., 2017; JANTZEN; GÖPEL; BEERMANN, 2013).

Conforme foi possível visualizar no Artigo III, os tratamentos contendo somente alginato e alginato + 5% de extrato apresentaram melhor desempenho na manutenção da vida útil do probiótico aplicado na polpa de morango quando submetido a simulação gastrointestinal.

6.3 VIABILIDADE DE *Lactobacillus casei* LC03 DURANTE O ARMAZENAMENTO

No Artigo II, realizou-se o armazenamento do probiótico livre e nas micropartículas dos diferentes tratamentos em três temperaturas distintas (25, 7 e -18°C), a fim de se selecionar uma matriz alimentícia que permaneça nessa mesma condição de armazenamento para a aplicação das micropartículas. Ao se comparar os resultados de viabilidade dos probióticos livres e microencapsulados nas três temperaturas, verificou-se que todos os tratamentos das micropartículas, inclusive a cultura livre, se mantiveram viáveis até o final dos 90 dias de armazenamento na temperatura de -18°C, o que não ocorreu na temperatura de 25°C, onde nenhum tratamento se manteve viável por 90 dias e na temperatura de 7°C, em que somente os tratamentos ALG e A5 permaneceram viáveis até o final do experimento. Além disso, na temperatura de -18°C, aos 90 dias de armazenamento, as contagens probióticas se mostraram mais elevadas em comparação com todos os outros tratamentos nas demais temperaturas. O congelamento é uma forma eficaz de conservação de diversas matrizes, pois atua inibindo de forma total ou parcial os principais fatores de alterações em alimentos: atividade enzimática, metabólica e microbiana, tanto de tecidos animais, quanto vegetais (ORDÓÑEZ, 2005).

Com os resultados da viabilidade probiótica durante o armazenamento, avaliados no Artigo II, chegou-se a conclusão de que a melhor opção de matriz alimentícia para a aplicação das micropartículas desenvolvidas nesse estudo seria um produto que permaneça armazenado sob congelamento. Em união a isso, com o propósito de ampliar o mercado de produtos probióticos para públicos vegetarianos/veganos, intolerantes a lactose e/ou ao glúten, foi elaborada a polpa de morango para aplicação das micropartículas.

No Artigo III, após aplicação das micropartículas, a polpa de morango contendo o probiótico livre ou microencapsulado foi armazenada congelada (-18°C) por 60 dias. A maior viabilidade dos probióticos foi alcançada na polpa que continha as micropartículas do tratamento AM (6,929 log UFC g^{-1}), seguida da polpa contendo o tratamento AME5 (6,161 log UFC g^{-1}). No decorrer do período de armazenamento danos celulares podem ser causados nos probióticos a partir da oxidação de lipídios da membrana, que levam a perda de sua

viabilidade (TEIXEIRA; CASTRO; KIRBY, 1995; YONEKURA et al., 2014). Os compostos bioativos presentes na casca de cebola roxa possuem alto poder antioxidante (CHIEW et al., 2014), que podem auxiliar na manutenção da vida útil dos probióticos durante seu armazenamento em um produto. Com a concentração de 5% de extrato (AME5) foi possível obter um equilíbrio na micropartícula adicionada na polpa de morango, visto que o extrato cumpriu com seu poder antioxidante ao mesmo tempo que, nessa concentração, não exerceu potencial efeito antimicrobiano ao probiótico *L. casei* LC03. Além disso, o tratamento AME5 fornece compostos bioativos/antioxidantes à dieta a partir da presença do extrato na cápsula, o que não ocorre no tratamento AM.

7. CONCLUSÕES

A principal conclusão do presente trabalho é que a microencapsulação propiciou a proteção do probiótico *L.casei* LC03, inclusive, na polpa de morango congelada; por haver a necessidade de se formular uma barreira de proteção, que impeça o contato entre o probiótico e a matriz alimentar, para que sua viabilidade durante armazenamento e consumo seja garantida. Além disso, o uso de extrato de casca de cebola roxa (*Allium cepa* L.) nas micropartpiculas é um meio efetivo de estender a viabilidade dos probióticos a partir do seu efeito prebiótico e antioxidante, gerando ainda, um produto polifuncional pela união dos compostos bioativos presentes no morango, com os probióticos e o extrato antioxidante de casca de cebola roxa. No entanto, para que isso seja possível, avaliar qual a melhor concentração do extrato para adição nas micropartículas foi imprescindível, uma vez que ao se utilizar concentrações superiores a 20% o extrato apresentou alto poder antimicrobiano frente ao probiótico. Por fim, as expectativas desse trabalho, de desenvolvimento de um novo produto probiótico sem glúten, sem lactose e vegano foram alcançadas com sucesso.

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