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MICROENCAPSULAÇÃO DE *Bifidobacterium* BB-12 POR GELIFICAÇÃO IÔNICA INTERNA: ESTUDO DA PRODUÇÃO, CARACTERIZAÇÃO E VIABILIDADE

DISSERTAÇÃO DE MESTRADO

Augusto Tasch Holkem

Santa Maria, RS, Brasil

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MICROENCAPSULAÇÃO DE *Bifidobacterium* BB-12 POR GELIFICAÇÃO IÔNICA INTERNA: ESTUDO DA PRODUÇÃO, CARACTERIZAÇÃO E VIABILIDADE

Augusto Tasch Holkem

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Mestre em Ciência e Tecnologia dos Alimentos.

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos
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MICROENCAPSULAÇÃO DE *Bifidobacterium* BB-12 POR GELIFICAÇÃO IÔNICA INTERNA: ESTUDO DA PRODUÇÃO, CARACTERIZAÇÃO E VIABILIDADE

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Os benefícios proporcionados pelos probióticos ao organismo humano têm proporcionado sua adição a diversos produtos, difundindo seu consumo. No entanto, devido a vários fatores como o armazenamento em baixas temperaturas, acidez e a passagem pelo trato gastrointestinal humano prejudicam a viabilidade destes microrganismos. A microencapsulação vem como alternativa de proteção destes probióticos até o intestino humano. O objetivo geral deste trabalho foi desenvolver microcápsulas probióticas de *Bifidobacterium* BB-12 por gelificação iônica interna na forma úmida e liofilizada. Além disto, foi analisado a sobrevivência dos probióticos sob condições gastrointestinais simuladas, tolerância "in vitro" quando inoculados em diferentes soluções de pH (4.5, 6.0 e 7.5) e viabilidade durante armazenamento sob diferentes temperaturas (-18, 7 e 25 °C) em diferentes tempos por 120 dias. Além da morfologia, diâmetro médio e caracterização físico-química das micropartículas. Sob as condições de 1.5% de alginato de sódio, 190 ×*g* de velocidade de rotação e 1.5% de emulsificante, as micropartículas apresentaram um diâmetro médio de 55 µm e uma eficiência de encapsulação superior a 90%. Em relação aos testes simulando as condições gastrointestinais, tanto as microcápsulas úmidas como as liofilizadas foram resistentes, apresentando uma contagem de 12.93 e 11.13 log UFC g^{-1} respectivamente, estando dentro dos padrões exigidos pela legislação brasileira para que ocorram os benefícios exercidos pelos probióticos. Tanto as microcápsulas úmidas como as liofilizadas apresentaram boa proteção em solução ácida (pH 4.5) e liberação total dos probióticos em solução fracamente básica (pH 7.5). A viabilidade das microcápsulas úmidas, foi mantida durante 75 dias à temperatura ambiente, sendo que houve uma redução de 6.74 log UFC g^{-1} ao longo do armazenamento devido a atividade metabólica existente, resultando assim na morte de células e perda de viabilidade celular. Comparado as outras temperaturas de armazenamento, na temperatura de refrigeração houve maior redução, que foi de 10.52 log UFC g^{-1}. Enquanto, que no congelamento apresentou os melhores resultados com uma viabilidade probiótica de 7.31 log UFC g^{-1} após os 120 dias. Analisando as microcápsulas liofilizadas, a temperatura ambiente ocasionou uma viabilidade probiótica até os 60 dias. Entretanto, as temperaturas de refrigeração e congelamento acarretaram em micropartículas viáveis por 120 dias de estocagem. Os resultados da caracterização físico-química e eficiência de encapsulação indicaram uma estabilidade das micropartículas com alta eficiência, facilitando a incorporação em produtos alimentícios.

Palavras-chave: Microencapsulação. *Bifidobacterium* BB-12. Gelificação iônica interna.

ABSTRACT

Master Dissertation
Post-Graduate Program in Food Science and Technology
Federal University of Santa Maria

MICROENCAPSULATION OF *Bifidobacterium* BB-12 BY INTERNAL IONIC GELATION: STUDY OF PRODUCTION, CHARACTERIZATION AND VIABILITY

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Place and Date: Santa Maria, January 8th, 2016.

The benefits provided by probiotics to the human body have provided their addition to various products, spreading their consumption. However, due to various factors such as storage at low temperatures, acidity and the passage through the human gastrointestinal tract undermine the viability of these organisms. Microencapsulation is alternative for the protection of these probiotics to the human intestine. The aim of this study was to develop probiotic microcapsules *Bifidobacterium* BB-12 by internal ionic gelation in the wet and freeze-dried form. Moreover, it was analyzed the survival of probiotics under simulated gastrointestinal conditions, tolerance "in vitro" when inoculated at different pH solutions (4.5, 6.0 and 7.5) and viability during storage at different temperatures (-18, 7, 25 °C) at different times for 120 days. In addition to the morphology, mean diameter and physicochemical characterization of microparticles. Under the conditions of 1.5% sodium alginate, 190 ×g rotation speed and 1.5% emulsifier, the microparticles had a mean diameter of 55 µm and an encapsulation yield greater than 90%. In relation to tests simulating gastrointestinal conditions, both the moist microcapsules as lyophilized were resistant, with microbial counts of 12.93 and 11.13 log CFU g^{-1} respectively, and these are within the standards required by Brazilian law to occur benefits exercised by probiotics. Both moist microcapsules as lyophilized showed good protection in acidic solution (pH 4.5) and total liberation of probiotics in weakly basic solution (pH 7.5). The viability of wet microcapsules was maintained for 75 days at room temperature, and there was a reduction of 6.74 log CFU g^{-1} over existing storage due to metabolic activity, thus resulting in cell death and loss of cell viability. Compared to other storage temperatures, the refrigeration temperature was further reduction, which was 10.52 log CFU g^{-1}. While, in the freezing showed the best results with a probiotic viability of 7.31 log UFC g^{-1} after the 120 days. Analyzing the lyophilized microcapsules at room temperature caused a probiotic viability by 60 days. However, refrigeration temperatures and freeze resulted in viable microparticles for 120 days of storage. The results of the physico-chemical characterization indicated encapsulation yield and stability of the microparticles with high efficiency, to facilitate incorporation into food products.

Keywords: Microencapsulation. *Bifidobacterium* BB-12. Internal ionic gelation.

LISTA DE ILUSTRAÇÕES

REVISÃO BIBLIOGRÁFICA

MANUSCRITO I

MANUSCRITO II

MANUSCRITO III

LISTA DE TABELAS

MANUSCRITO I

MANUSCRITO II

MANUSCRITO III

LISTA DE ABREVIATURAS E SIGLAS

$\times g$	– Força centrífuga relativa
BB-12	– *Bifidobacterium animalis* subsp. *lactis* BB-12
Bifidobacterium BB-12	– *Bifidobacterium animalis* subsp. *lactis* BB-12
$CaCO_3$	– Carbonato de cálcio
MRS	– Ágar padrão de crescimento de *Lactobacillus* (DE MAN, ROGOSA, SHARPE, 1960).
RPM	– Rotação por minuto
Tween 80	– Polissorbato 80
UFC	– Unidade formadora de colônias

SUMÁRIO

1 INTRODUÇÃO

Os alimentos funcionais caracterizam-se por oferecer inúmeros benefícios à saúde, pois além do seu valor nutritivo inerente à sua composição química, os mesmos podem desempenhar um papel potencialmente benéfico na prevenção de doenças crônicas, degenerativas e cardiovasculares dentre muitas outras. Em vista disso, o mercado de alimentos funcionais está crescendo em todo o mundo e novos produtos estão sendo lançados continuamente. No entanto, estes novos produtos funcionais só serão viáveis se os consumidores estiverem dispostos a aceitá-los como parte de sua dieta diária (BIGLIARDI & GALATI, 2013; LAHTEENMAKI, 2013; LAYMAN, 2014).

A maioria dos produtos probióticos são categorizados como alimentos funcionais e representam parte significativa da mesma. Porém, o maior problema encontrado pelas indústrias na aplicação de bactérias probióticas em alimentos está relacionado à viabilidade e manutenção destas culturas (KOŁOŻYN-KRAJEWSKA & DOLATOWSKI, 2012; LALIČIĆ-PETRONIJEVIĆ et al., 2015; OLNOOD et al., 2015).

Os probióticos devem ser seguros e estar em quantidades adequadas no momento do consumo (FAO/ WHO, 2001; SINGH et al., 2011). Porém, em algumas condições como baixo pH, alta acidez, presença de sais biliares, choques térmicos causados por atomização ou congelamento, contato com o oxigênio molecular para microrganismos anaeróbicos e o próprio trato gastrointestinal humano representam um ambiente desafiador para a sobrevivência desses microrganismos (CHEN & MUSTAPHA, 2012; CHEOW et al., 2014; NAG & DAS, 2013; SETTACHAIMONGKON et al., 2015). Portanto, a concentração destas bactérias deve ser elevada no produto para que a ação de colonizar e proliferar aconteça e o efeito benéfico no intestino ocorra (DUNCAN & FLINT, 2013; TRIPATHI & GIRI, 2014).

Esses obstáculos podem ser transpostos através do processo de microencapsulação e imobilização. Por meio das propriedades de liberação controlada finamente ajustadas, a microencapsulação deixa de ser somente um método de agregação de substâncias a uma formulação alimentícia, tornando-se uma fonte de ingredientes totalmente novos com propriedades únicas (MACIEL et al., 2014; MIRZAEI et al., 2012). Portanto, fazem-se necessários mais estudos de novas estratégias para a manutenção destes microrganismos, sendo a microencapsulação um método promissor para a proteção destes probióticos.

A gelificação iônica interna é uma técnica inovadora para a produção de micropartículas de polissacarídeo iônico. Este método é rápido, facilmente escalonado e

utiliza materiais biocompatíveis (CAI et al., 2014). Comparado com extrusão, a emulsificação/gelificação iônica interna é mais viável no controle do tamanho de partículas e produção em larga escala (BHANDAR & DEETH, 2003; HOESLI et al., 2011; KRASAEKOOPT, LUPO et al., 2015).

Com a gelificação interna o problema da aglomeração das micropartículas não ocorre como encontrado na técnica de gelificação externa, devido à liberação lenta dos íons Ca^{2+} a partir de fontes de cálcio insolúveis em vez da adição de cloreto de cálcio solúvel diretamente. Portanto, esta técnica produz microcápsulas com uma estrutura interna desenvolvida e uma liberação controlada ao longo do tempo com diâmetros menores que 100 µm. A produção à frio e baixo custo de materiais faz desta técnica um potencial para emprego em escala industrial na encapsulação de ingredientes ativos, principalmente na microencapsulação de probióticos para aplicação em alimentos (WESTERIK et al., 2015).

1.1 Objetivos

1.1.1 Objetivo geral

Microencapsular *Bifidobacterium* BB-12 por gelificação iônica interna avaliando sua viabilidade e resistência com potencial aplicação em alimentos.

1.1.2 Objetivos específicos

- Otimizar o processo de microencapsulação de *Bifidobacterium* (BB-12) variando as diferentes concentrações de polissacarídeo, emulsificante e velocidades de emulsificação (rotações).
- Utilizar o método de microencapsulação por gelificação iônica interna avaliando as suas condições de liberação controlada;
- Avaliar a estabilidade das microcápsulas úmidas e liofilizadas em diversas condições de pH, simulando as condições do trato gastrointestinal humano;

- Caracterizar a composição físico-química das micropartículas;
- Avaliar a forma, diâmetro e distribuição das microcápsulas úmidas e liofilizadas;
- Acompanhar a viabilidade da cultura probiótica durante o período de tempo de 120 dias para as microcápsulas úmidas e liofilizadas nos diferentes tratamentos em temperatura ambiente, de refrigeração e de congelamento.

2 REVISÃO DA LITERATURA

2.1 Probióticos

Os probióticos são definidos como microrganismos vivos, que conferem benefícios à saúde do hospedeiro quando administrado em quantidades adequadas (FAO/WHO, 2001). Estas quantidades são variáveis conforme o país. Por exemplo, nos Estados Unidos (EUA) desenvolveu-se um padrão que requer pelo menos 10^8 UFC/g. De acordo com a Agência Nacional de Vigilância Sanitária – ANVISA (BRASIL, 2002), a quantidade mínima diária de probióticos viáveis que devem ser ingeridos para efeitos terapêuticos é de 10^8 a 10^9 UFC, o que equivale ao consumo de 100 g de produto contendo entre 10^6 a 10^7 UFC de microrganismos. Valores menores podem ser aceitos, desde que comprovada a sua eficiência, mediante a apresentação de laudo de análise do produto que comprove a quantidade mínima viável até o final do prazo de validade. A quantidade do probiótico em unidades formadoras de colônia (UFC), contida na porção diária do produto pronto para consumo, deve ser declarada no rótulo (CHAMPAGNE et al., 2011; RAD et al., 2012).

As características para um bom probiótico são as seguintes: 1) A cultura pode ser produzida em escala industrial; 2) A cultura tolera o ambiente intestinal do hospedeiro; 3) A cultura pode sobreviver durante a produção, armazenamento e exercer efeitos sobre a saúde quando consumido (FULLER, 1989; SINGH et al., 2011). Estes microrganismos devem ser caracterizados de origem humana e de uso seguro para o hospedeiro. A comprovação dos seus efeitos fisiológicos benéficos é necessária. Além disso, deve ser efetivamente capaz de aderir ao tecido alvo (OYETAYO & OYETAYO, 2005; PICARD et al., 2005).

Os efeitos dos probióticos na saúde são inúmeros (FERREIRA, 2009; SALMINEN et al., 1998; SINGH et al., 2011), tais como:

- melhora na digestibilidade de alguns nutrientes dietéticos, tais como proteínas e gorduras;
- aliviar os sintomas de intolerância a lactose;
- modulação de reações alérgicas através do desenvolvimento da função de barreira da mucosa;
- tratamento de muitos tipos de diarreia tanto de adultos como em crianças;

- preservação da integridade do intestino e combate a doenças inflamatórias como síndrome do intestino irritável, colite e doença hepática alcoólica;
- aumento nos níveis de citocinas e imunoglobulinas e consequentemente aumento da resposta imune;
- Inibição de patógenos, reduzindo a chance de infecção.

Os principais microrganismos probióticos empregados nos alimentos pertencem ao grupo heterogêneo de bactérias ácido-lácticas. São do gênero *Lactobacillus* como, por exemplo*: Lactobacillus acidophilus* e *Lactobacillus casei*. E outro grande grupo de bactérias usadas como probióticos pertencem ao gênero *Bifidobacterium* como: *B. bifidum e B. subsp. lactis* (AMARA & SHIBL, 2015; HOMAYOUNI et al., 2008; KAILASAPATHY & SULTANA, 2003; TERGUT & CAKMAKCI, 2009).

Os tipos de microrganismos usados como probióticos tem aumentado devido ao aumento da investigação sobre o assunto, bem como pelo aumento das descobertas e identificação de novas cepas, que podem ser utilizadas como probióticos. Com as novas pesquisas, a composição da flora microbiana deve ser atualizada e os dados publicados sobre probióticos ganhará mais destaque (GOSÁLBEZ & RAMÓN, 2015; PATEL, SHUKLA & GOYAL, 2015).

2.2 Gênero *Bifidobacterium*

As bactérias do gênero *Bifidobacterium* são bastonetes anaeróbios, Gram-positivas, não formam esporos e não apresentam mobilidade. Fermentam açúcares produzindo principalmente ácido acético e lático, não formando gás carbônico. O pH ótimo de crescimento está entre 6,0 e 7,0, praticamente sem crescimento abaixo do pH 5,0 e acima de 8,0. A temperatura ótima de crescimento está entre 37 e 41 °C, com máximo entre 43 e 45 °C e praticamente sem crescimento abaixo de 25 e 28 °C (GOMES & MALCATA, 1999; ROY, 2005).

Bifidobacterium BB-12 é uma bactéria de catalase-negativa em forma de haste. Foi isolada no banco de células da empresa Chr. Hansen, em 1983 e foi considerado como pertencendo à espécie de *Bifidobacterium bifidum*. Técnicas de classificação molecular moderna reclassificaram BB-12 como *Bifidobacterium animalis* e logo se incluiu a subespécie *lactis.* Hoje, o BB-12 é dessa forma classificado como *Bifidobacterium animalis* subsp. *lactis.*

Ainda que ao longo dos anos a nomenclatura tenha mudado, a estirpe BB-12 não foi alterada (GARRIGUES et al., 2010; JUNGERSEN et al., 2014). O *B. animalis* subsp. *lactis* é a espécie mais utilizada de *Bifidobacterium,* devido à sua resistência às condições de estresse encontradas no produto alimentar e durante passagem gastrointestinal comparado a outras espécies probióticas (MATTO et al., 2006).

As bifidobactérias são um dos maiores grupos de bactérias no cólon, o qual constitui mais de 25% do total da população intestinal adulta e 95% em recém-nascidos. Produzem numerosos efeitos benéficos ao hospedeiro, tais como: redução do pH produzindo um efeito bactericida; participação na produção de vitaminas do complexo B e influência na resposta imune; fermentação de substratos, resultando na produção de ácidos graxos de cadeia curta (AGCC); diminuição dos níveis séricos de amônia pela fermentação de proteínas e possui uma grande eficácia na prevenção e tratamento de um grande espectro de distúrbios gastrointestinais, tais como doenças do trânsito intestinal, infecções e adenomas no colón e câncer (BENGMARK, 1998; BERG et al., 2012). Têm sido muito utilizados em produtos lácteos fermentados, pois apresentam alta capacidade de sobrevivência gastrointestinal e exibem propriedades probióticas no intestino (ALBENZIO et al., 2013; CHAVES & GIGANTE, 2016; DONKOR et al., 2007; PICARD et al., 2005).

2.3 Microencapsulação

A microencapsulação é um processo no qual um material ou mistura (líquido, gás ou sólido) é revestido ou aprisionado no interior de uma cápsula, que pode liberar o seu conteúdo no local ou momento ideal a taxas controladas sob condições específicas, evitando o efeito de uma exposição inadequada (DESAI & PARK, 2005; LIU et al., 2002; PICOT & LACROIX, 2004; RISCH, 1995). O material que vai ser revestido normalmente é denominado por material do núcleo, ativo ou fase interna e o material que forma a cápsula, encapsulante, cobertura ou parede (AZEREDO, 2005; POSHADRI & KUNA, 2010).

As microcápsulas devem ser projetadas para liberar o núcleo para o ambiente externo no momento desejado. Diferentes tipos de “gatilho” podem ser utilizados para a liberação do material encapsulado tais como alteração de ruptura mecânica, pH, ação enzimática, temperatura, força osmótica, entre outros. A liberação no momento e no lugar adequado é extremamente importante no processo de encapsulamento, expandindo a aplicação dos

compostos de interesse, melhorando a eficácia e reduzindo a dose de aditivos (GOUIN, 2004; SILVA et al., 2014).

A denominação de micropartículas é usual para as partículas sólidas que têm diâmetro de 1 a 1000 µm. Essas podem ser classificadas em microcápsulas (sistema reservatório) ou microesferas (sistema matriz) conforme a Figura 1. Nesse caso, as microcápsulas possuem o material ativo rodeado por um invólucro de polímero, enquanto nas microesferas a fase interna é molecularmente dissolvida ou heterogeneamente dispersa no polímero da matriz (FÁVARO-TRINDADE et al., 2008; NORDSTIERNA et al., 2010; PIMENTEL et al., 2007).

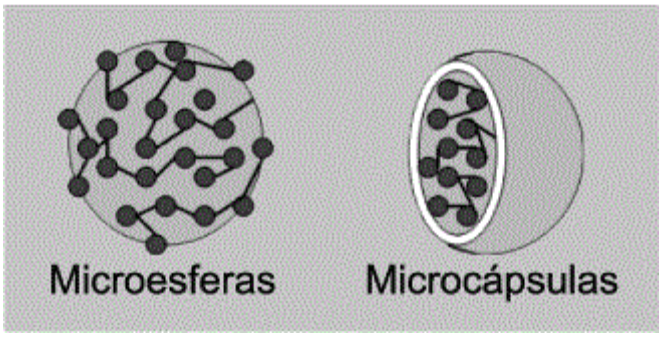

Figura 1 – Esquema ilustrativo diferenciando os dois tipos de micropartículas.
Fonte: Adaptado de PIMENTEL et al., 2007.

O material encapsulante é selecionado em função das propriedades físicas e químicas do agente ativo, da aplicação pretendida e do método utilizado para formar as micropartículas. Existem diversos tipos de materiais encapsulantes, como por exemplo, o alginato de cálcio, proteínas do soro do leite e gomas. A escolha do agente encapsulante depende de uma série de fatores, entre eles: a não reatividade com o material a ser encapsulado durante o processo e estocagem; o processo utilizado para a formação da microcápsula; o mecanismo de liberação do material encapsulado; suas propriedades reológicas; a habilidade de dispersar ou emulsificar; a capacidade de prover a máxima proteção para o material a ser encapsulado contra condições desfavoráveis, e ser economicamente viável (DESAI & PARK, 2005).

O alginato é um dos materiais de encapsulação mais amplamente utilizados, sendo um heteropolissacarídeo linear composto de ácido β-D-manurônico e ácido α-L-gulurônico (GACESA, 1988). Entre os sais de alginato mais empregados, se encontra o alginato de sódio, devido a sua alta solubilidade em água fria e características de transição de solução para gel de forma instantânea quando em contato com íons cálcio (FUNAMI et al., 2009). Vários métodos de microencapsulação de probióticos com alginato foram relatadas, incluindo a

secagem por pulverização, revestimento por pulverização, extrusão, emulsificação e coacervação (CHAMPAGNE & FUSTIER, 2007; GOUIN, 2004).

2.4 Métodos de microencapsulação

De acordo com Jamekhorshid et al. (2014), atualmente, há inúmeros métodos de microencapsulação patenteados e cada vez mais em ascendência. É previsível que esse número continue crescendo à medida que forem surgindo novos materiais encapsulantes e novos princípios ativos que demandem processamentos específicos para a encapsulação. Dependendo da técnica utilizada são formadas diferentes microcápsulas com morfologia, estrutura e propriedades térmicas.

A escolha do método mais adequado depende do tipo do material ativo, da aplicação que será dada a microcápsula, das propriedades químicas e físicas do núcleo e da parede, da escala de produção e do custo, do tamanho das partículas requeridas e do mecanismo de liberação desejado para a sua ação. A tecnologia de microencapsulação é usada para proporcionar uma estabilidade do ingrediente ativo até o seu local destinado. Ter um melhor entendimento das interações moleculares e das propriedades do ingrediente ativo e da composição do material é crucial para a criação de um sistema de encapsulação eficaz (SUAVE et al., 2006; VASISHT, 2014).

Os métodos mais comumente utilizados para o aprisionamento de substâncias são (LAM & GAMBARI, 2014; SANTOS et al., 2001):

1) Processos químicos: inclusão molecular e polimerização interfacial;
2) Processos físico-químicos: coacervação simples e complexa, emulsificação seguida de evaporação do solvente, pulverização em agente formador de reticulação e envolvimento lipossômico.
3) Processos físicos: *spray cooling*, *spray drying*, pulverização em banho térmico, leito fluidizado, extrusão em centrífuga com múltiplos orifícios, cocristalização e liofilização;

Várias tecnologias podem ser aplicadas para encapsulamento probiótico e cada uma delas fornece microcápsulas com características diferentes em termos de tamanho de partículas e do tipo de cápsula (BURGAIN et al., 2011). Por exemplo, a emulsificação permite a produção de uma ampla gama de tamanho de partícula a partir de 0,2 µm para 5000

µm, porém a extrusão dá uma gama de tamanho menor, mas não fornece partículas inferiores a 300 µm. Na Figura 2 observa-se os diferentes tipos de partículas obtidas por cada um dos métodos.

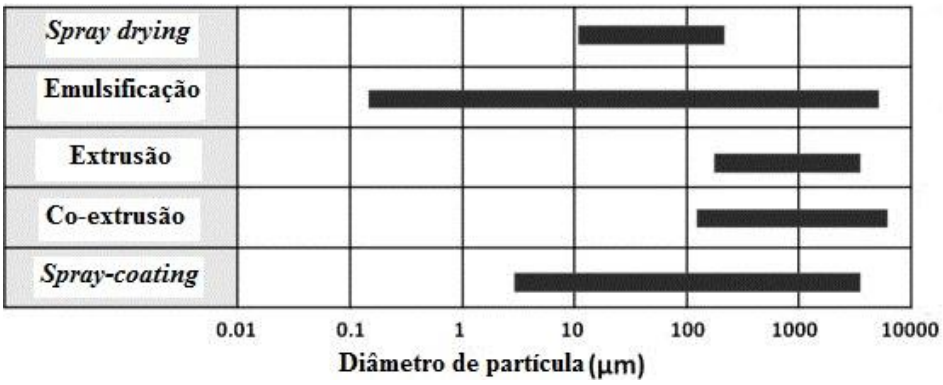

Figura 2 – Tecnologias de encapsulação de probióticos: diâmetro de partícula proveniente de cada técnica.
Fonte: Adaptado de BURGAIN et al., 2011.

Segundo Rokka & Rantamäki (2010), a microencapsulação provou ser um dos métodos mais eficientes para manter alta estabilidade e viabilidade das bactérias probióticas, uma vez que protege os probióticos das condições adversas do trato digestivo e também durante o processamento e armazenamento do alimento (CHAMPAGNE & FUSTIER, 2007). Ela compreende um conjunto de diversas técnicas que permitem o desenvolvimento de formulações, em que o seu conteúdo é protegido e sua liberação pode ser modificada com o objetivo de atuar num determinado local, por um determinado período de tempo e uma velocidade específica (SUAVE et al., 2006).

2.5 Emulsificação/gelificação iônica interna

A produção de micropartículas através da técnica de gelificação iônica é um método de encapsulação rápido, que possui formulação e procedimento simples, de baixo custo e têm sido aplicado nas áreas de biotecnologia, medicina e na indústria de alimentos. Está baseada na capacidade dos polieletrólitos de fazerem ligações cruzadas na presença de contra-íons, formando uma malha tridimensional. Por essa técnica, uma solução polimérica carregada

negativamente forma ligações cruzadas (*cross-linking*) com cátions de baixa massa molecular, formando hidrogéis (COSTA, 2014; RÉ, 2010).

Neste método as cápsulas são formadas a partir de duas etapas: a dispersão de uma fase aquosa, contendo o composto ativo e uma suspensão polimérica, dentro de uma fase orgânica, como óleo, resultando em uma emulsão de água em óleo. Um ácido é então adicionado ao meio que se propaga ao longo da fase contínua para as gotículas. Com isso, ocorre a diminuição do pH e íons Ca^{+2} são liberados permitindo o empilhamento dos blocos G no polímero de alginato formando o modelo "*egg-box*" (Figura 3) aprisionando o composto ativo (CHAN et al., 2002; O'DONNELL & MCGINITY, 1997; PONCELET, 2001).

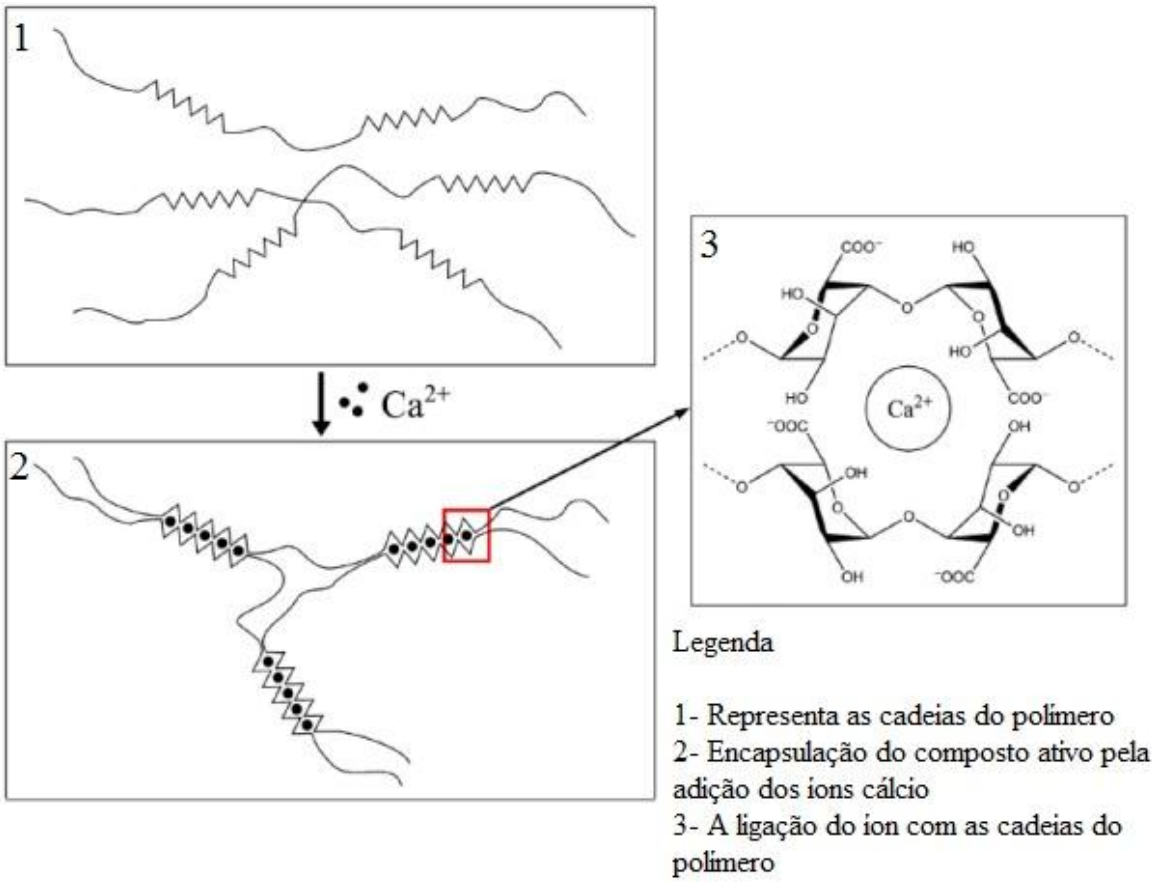

Figura 3 - Modelo "*egg-box*" para formação de gel de alginato com íons cálcio.
Fonte: PAQUES, 2015

Para o preparo das micropartículas pelo método de emulsificação/gelificação interna é necessário o conhecimento da concentração de alginato, velocidade de rotação na emulsão e concentração de emulsificante (AHMED et al., 2013). A natureza e a concentração destes fatores influenciam nas propriedades das partículas formadas pela gelificação como o volume, índice de difusão dos solutos para dentro ou fora da matriz, cinética de formação do gel e

também a porosidade e estabilidade das cápsulas (MARTIN et al., 2013). Para aplicações em alimentos, o diâmetro médio das micropartículas, é uma das características mais importantes, sendo que estas devem ser suficientemente pequenas, para evitar um impacto sensorial negativo, sendo o tamanho desejável de aproximadamente 100 μm (COSTA et al., 2015; HEIDEBACH, FÖRST, & KULOZIK, 2009).

Na área de alimentos, os hidrocolóides mais utilizados são o alginato e a pectina. São polieletrólitos naturais e com grande capacidade de formação de hidrogéis possuindo uma característica vantajosa de biocompatibilidade com membranas e ausência de tensão interfacial com fluidos (CHAN et al., 2006; TEIXEIRA et al., 2014; VOS et al., 2010). Algumas desvantagens são atribuídas a micropartículas de alginato. Por exemplo, os alginatos são sensíveis a condições muito ácidas pois perdem a sua estabilidade mecânica nesses ambientes. Além disso, o gel de alginato é formado na presença de íons de cálcio, assim, a sua integridade é deteriorada quando sujeita a íons monovalentes ou quelantes (fosfatos, lactatos e citratos). Os defeitos mencionados podem ser resolvidos através da mistura de alginato com outros compostos de polímeros, revestimento do alginato com substâncias diferentes ou fazer alguma modificação estrutural do alginato (KRASAEKOOPT et al., 2003).

A emulsificação tem sido aplicada com sucesso na microencapsulação de bactérias ácido-láticas (KRASAEKOOPT, BHANDARI & DEETH, 2003). Wang et al., (2014) desenvolveram microcápsulas de alginato com proteína de grão de bico utilizando a tecnologia de emulsificação. As partículas ofereceram boa proteção para *Bifidobacterium adolescentis* no suco gástrico sintético. Os grânulos produzidos apresentaram diâmetro superior que 100 μm. Assim, não houve percepção de efeitos adversos sobre os atributos sensoriais deste ingrediente em alimentos por parte dos consumidores. O estudo sugere que as microcápsulas de proteína de grão de bico e alginato poderiam servir como um veículo adequado para uso de probiótico destinado para aplicações alimentares.

Diversos estudos indicam que a eficiência de encapsulação se mostra elevada na técnica de emulsificação/gelificação interna. A levedura probiótica, *S. boulardii* foi encapsulada em micropartículas de alginato de cálcio preparadas pelo método de emulsificação com inulina e mucilagem como materiais de revestimento. As células encapsuladas apresentaram eficiência de encapsulação superior a 70% durante 35 dias de armazenamento (ZAMORA-VEGA et al., 2012). Em outro estudo realizado por Özer et al. (2008) que encapsularam as culturas probióticas de *B. bifidum* e *L. acidophilus* por extrusão e emulsificação retrataram uma alta viabilidade durante os 90 dias de armazenamento. Takei et al. (2008) que microencapsularam *S. cerevisiae* por emulsificação obtiveram eficiência de

encapsulação superior a 50% com a diminuição da proporção em peso da fase aquosa e da fase oleosa.

Essa técnica tem sido frequentemente empregada, tendo em vista a simplicidade dos procedimentos envolvidos na obtenção das partículas e as possibilidades de modulação das características físicas e físico-químicas das partículas por meio da escolha dos componentes da formulação e das condições de preparação. Indica uma boa reprodutibilidade de trabalho com pouca variação das micropartículas e maior controle de diâmetro, aplicável em maior escala. O custo de utilização desta técnica é relativamente baixo, devido a utilização de materiais biocompatíveis e baratos. E os efluentes gerados devido a retirada da fase oleosa no processo de encapsulação, possivelmente podem ser tratados e utilizados na produção de biocombustíveis como etanol e biodiesel (LUPO et al., 2014; MUKAI-CORRÊA et al., 2004; PONCELET et al., 1992).

2.6 Algumas aplicações da encapsulação de probióticos e outras substâncias na indústria de alimentos

A aplicação mais conhecida da encapsulação em alimentos é a proteção de compostos de aroma, que podem ser perdidos por evaporação, oxidação ou interações com outros compostos. Uma grande preocupação é que os compostos de aroma sejam liberados de forma rápida e efetiva quando o alimento entra em contato com a boca, do contrário a encapsulação não teria sentido. Assim, geralmente são utilizados materiais de parede bastante hidrossolúveis, como amido e dextrinas (AZEREDO, 2005; ROMO-HUALDE, et al., 2012)

Alguns trabalhos foram realizados envolvendo a encapsulação de corantes. As fortes restrições impostas pelo mercado ao uso de corantes sintéticos têm motivado sua crescente substituição por pigmentos naturais. A encapsulação tem aumentado consideravelmente a estabilidade de carotenóides, antocianinas e betalaínas (BERG et al., 2012; DONHOWE et al., 2014; KHAZAEI et al., 2014).

Os óleos essenciais estão sujeitos à oxidação, isomerização e polimerização e interagem facilmente com outros componentes voláteis, alimentos e embalagens. Desta forma poderão sofrer um decréscimo da intensidade do aroma e contribuir para o desenvolvimento de aromas estranhos. Por conseguinte, a sua retenção numa microcápsula sólida, inócua e de origem alimentar, irá facilitar a sua manipulação, assim como a preservação dos seus

constituintes durante um maior período de tempo (CARVALHO et al., 2009; GALLARDO et al., 2013; SILVA et al., 2014).

Entre outras substâncias que podem ser microencapsuladas como edulcorantes, acidulantes, minerais e vitaminas (COMUNIAN et al., 2013; ÇAM et al., 2014; ROCHA-SELMI et al., 2013; ZHU et al., 2014).

A utilização dos processos de microencapsulação de probióticos é uma alternativa promissora possibilitando a reutilização dos mesmos na produção de ácido láctico e produtos lácteos fermentados (ABDEL-RAHMAN et al., 2013; ESPIRITO-SANTO et al., 2014; KANMANI et al., 2011; PINTO et al., 2012); para protegê-los contra a presença de oxigênio (BATTINI et al., 2014; KENT & DOHERTY, 2014); contra o efeito bactericida do suco gástrico e outros meios ácidos (OKURO et al., 2013; PEDROSO et al., 2012); para aumentar a concentração de células em reatores, objetivando o aumento da produtividade (DOLEYRES & LACROIX, 2005); contra as baixas temperaturas (AMINE et al., 2014; SEMYONOV et al., 2010). E ainda, manter a viabilidade da cultura durante a estocagem do produto (AVILA-REYES et al., 2014; DIANAWATI et al., 2013; YING et al., 2013).

A aplicação de culturas probióticas normalmente ocorre em produtos de origem láctea, pois representa um ambiente ideal para a conservação dos microrganismos probióticos, tais como leites fermentados, sorvetes, vários tipos de queijo, alimentos para bebês, leite em pó, sobremesas lácteas congeladas, bebidas à base de soro de leite, creme de leite, leitelho e leite aromatizado (MOHAMMADI & MORTAZAVIAN, 2011). Todavia, tendo em vista a alta prevalência de intolerância à lactose, diferentes produtos probióticos não lácteos, tais como produtos à base de vegetais, produtos à base de cereais, sucos de frutas, produtos à base de soja, sobremesas à base de aveia, produtos de confeitaria, cereais matinais e alimentos para bebês têm sido desenvolvidos nos últimos anos (ANTUNES et al., 2013; NOORBAKHSH et al., 2013; SAAD, 2006).

4 MANUSCRITO I

Improvement the viability of probiotics encapsulated by emulsification/internal gelation*

ABSTRACT

Microencapsulation of *Bifidibacterium* BB-12 by emulsification/internal gelation is an technique with great potential for protection and improvement of viability of probiotics for use in food products. In this study, solid lipid microparticles containing *Bifidobacterium* BB-12 produced by emulsification using sodium alginate as wall material were investigated. An optimum condition for encapsulation was obtained using alginate concentration at 1.5% at rotational speed of 190 ×*g*, and emulsifier concentration of 1.5%, which produced microparticles with lower diameters (55 μm). The encapsulation yield (EY%) was also investigated, with optimum values above 99% efficiency and 13.98 ± 0.12 log CFU g^{-1} probiotic viability.

Keywords: probiotics; microencapsulation; internal ionic gelation; viability.

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1. Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer health benefits for the host (FAO/WHO, 2002). Lactic acid bacteria are the most important probiotics because of their beneficial effect mainly on the human gastrointestinal tract (Chaikham et al., 2012). These bacteria are gram-positive and usually live in anaerobic environments, but very vulnerable to adverse conditions such as temperature, pH, and oxygen, resulting in a significant reduction in the number of viable cells, thus impairing their positive effect on health (Burgain et al., 2011). Microencapsulation has proven to be an efficient technology for use in the food industry, protecting bacteria at its site of action (Cai et al., 2014).

Although a variety of probiotic encapsulation technologies has been studied, many disadvantages with respect to these different techniques have been reported by several authors. Microencapsulation by spray-drying can cause decreased viability of the microorganisms due to the high inlet air temperature and evaporation rates during the process, destroying the bacterial cytoplasmic membrane (Arslan et al., 2015). Furthermore, the external gelation/extrusion requires specific equipment such as syringes and needles, while other studies have also used vibrating nozzles, atomizing nozzle and atomizing disks to enhance extrusion operations. Although these systems reduce particle size according to the needle, they are expensive techniques (Prisco et al., 2015). Complex coacervation is another encapsulation method considered unstable due to different hydrogenionic potential values (pH). The complex coacervation process does not provide effective protection for the probiotics, as it provides a low resistance of the microparticles at pH values similar to the human stomach (Shoji et al., 2013).

Thus, the emulsification/internal gelation technique emerges as a viable alternative to ensure the protection and viability of probiotics. This consists of emulsifying an aqueous solution of alginate-active component and an insoluble or partially soluble salt of calcium in a non-aqueous phase accompanied by an emulsifier. An acid is then added to the medium that propagates along the continuous phase. The Ca^{2+} ions are released with decreasing pH, leading to gelation with consequent entrapment of active material (Song et al, 2013.). This technique has many advantages such as greater control and reducing particle diameter; production of microparticles in temperature ideal for probiotic viability; simplicity, quickness, and low cost for large-scale production, besides allowing the use of natural and inert polysaccharides (Ibarguren et al., 2012).

For the preparation of microparticles by emulsification/internal gelation method, it is necessary to know the alginate concentration, the emulsion rotational speed, and emulsifier concentration (Ahmed et al., 2013). The nature of these factors affects the distribution and the particle size and consequently the viability of the organism under study (Martin et al., 2013). For food applications, the average diameter of microparticles is one of the most important characteristics, since they must be sufficiently small to avoid a negative impact on sensory characteristics, with desired size of approximately 100 μm (Costa et al., 2015).

Therefore, this study aimed to optimize by response surface methodology the protection of *Bifidobacterium* BB-12 encapsulated by emulsification/internal gelation using smaller microparticle diameters obtained by different sodium alginate concentrations, rotational speeds, and emulsifier concentrations, in addition to propose a systematic study of encapsulation yield.

2. Material and methods

2.1. Materials

Sodium alginate was kindly provided by FMC BioPolymer (Campinas, Brazil). Canola oil (Liza®, Cargill, Mairinque, São Paulo, Brazil). $CaCO_3$ (calcium carbonate) was acquired by Neon Comercial Ltda (São Paulo, Brazil). Other products such as Tween 80 (Polysorbate 80), $C_2H_4O_2$ (Glacial Acetic Acid), $CaCl_2.2H_2O$ (Calcium Chloride Dihydrate) were acquired by Vetec Química Fina Ltda (Rio de Janeiro, Brazil).

2.2. Culture

The lyophilized culture of *Bifidobacterium animalis* subsp. *lactis* BB-12 was kindly supplied by Chr. Hansen (Valinhos, Sao Paulo, Brazil) with cell viability of about 14 log CFU g^{-1}. This culture was stored at -18 °C until the time of use.

2.3. Microencapsulation process

The production of microparticles was performed by the emulsification/internal gelation technique developed by Poncelet (1992) with modifications. For that, 40 mL sodium

alginate solution at different concentrations (1.16-2.84%) was mixed with 1g freeze-dried microorganism and 2 mL of $CaCO_3$ suspension (500 mM Ca^{2+}). This mixture was transferred to a round-bottom reactor (60 mm internal diameter and 90 mm height) and dispersed for 15 minutes in 200 mL canola oil containing different concentrations of Tween 80 (0.16-1.84%) at different rotational speeds (2.3-338 ×*g*) using an automatic stirrer MacroQuimis® (Diadema, Sao Paulo, Brazil) provided with a three-blade marine type propeller (8 mm thick and 42 mm internal diameter between the blades), forming a uniform emulsion water in oil. Under continued stirring, 40 mL canola oil containing 160 µL glacial acetic acid were added to the emulsion. After 5 minutes the stirring was stopped. The capsules were separated by addition of 300 mL of 0.05M $CaCl_2.2H_2O$. After complete separation of the microparticles to the aqueous phase, the oil was discarded and the microcapsules were filtered in sieve with porosity of 0.038 mm (400 mesh) and washed in distilled water at pH 4.0 until complete oil removal and stored at 4 °C for further analysis.

2.4. Enumeration of microencapsulated Bifidobacterium BB-12

The microencapsulated bacteria were released from the capsules according to the method of Sheu & Marshall (1993) with modifications. One gram of microparticles was resuspended in 9 mL phosphate buffer (0.1 M, pH 7.5) followed by homogenization in a stomacher for 7 minutes. To enumerate bifidobacteria, samples were serially diluted with peptone water (0.1 g 100 g^{-1}) and plated on modified MRS agar with addition of lithium chloride (0.1%) and L-cysteine (0.05%), according to Chr Hansen (1999). Plates were incubated under anaerobic environment in anaerobic jars with Anaerobac system (Probac, Sao Paulo, Brazil) at 37 °C for 72 h. After the incubation period, the probiotic viable cell was enumerated.

2.5. Encapsulation yield

The encapsulation yield (EY%) which is the microorganism survival rate during the microencapsulation process was calculated according to Eq. (1) as proposed by Martin et al. (2013).

$$EY\% = (N/N_0) \times 100 \qquad (1)$$

Where N is the number of viable cells (log CFU g^{-1}) released from the microparticles and N_0 is the number of viable cells (log CFU g^{-1}) in the cell concentrate for microencapsulation.

2.6. Evaluation of the mean diameter and size distribution of the microparticles

The size distribution of the microparticles was measured on the Mastersizer 2000 equipment (Malvern, Germany) using water as the dispersion medium. The amount of sample added varies from sample to sample, when reaching the obscuration range of 3 to 20% as recommended by the manufacturer.

2.7. Statistical Analysis

Response surface methodology was used to determine the optimal conditions for preparation of microparticles, as a function of three experimental factors: alginate concentration (1.16, 1.5, 2.0, 2.5 and 2.84% w/v), rotational speed (2.3, 21, 84.5, 190 and 338 ×*g*), and emulsifier concentration (0.16, 0.5, 1.0, 1.5 1.84% w/v). A five-level central composite design was used. The experimental design and the statistical analyses were carried out using the Statistica 7.0 software (Statsoft, Tulsa-OK, USA).

3. Results and discussion

3.1. Effect of concentration of sodium alginate on the diameter of microparticles

Table 1 shows the results of average diameters and the percentage of encapsulation yield. The average diameters of the microparticles ranged from 53.87 to 875.28 µm. The concentration of 1.5% alginate has proven to be the best overall performance when compared to the other concentrations studied. In this condition, there was the formation of morphologically regular microparticles with an average diameter of 55 µm and an appropriate size distribution. When concentrations of 1.16% and above 1.5% alginate were used, the formation of microparticles with diameters varying from 119.60 to 875.28 µm was observed, which increased the size distribution range.

Table 1
Coded matrix of the alginate concentration, rotational speed and emulsifier concentration on the diameter microspheres (µm) and encapsulation yield (%)

Run	X_1 (%)	X_2 (×*g*)	X_3 (%)	Diameter (µm)	EY (%)
1	+1	-1	-1	306.88	71.57
2	+1	+1	+1	208.93	86.00
3	+1	-1	+1	260.14	69.78
4	+1	+1	-1	244.75	71.35
5	-1	+1	-1	54.82	81.00
6	-1	+1	+1	53.87	99.85
7	-1	-1	+1	59.10	88.28
8	-1	-1	-1	54.81	73.28
9	-1.68	0	0	119.60	65.50
10	+1.68	0	0	875.28	83.85
11	0	0	0	249.70	72.07
12	0	-1.68	0	207.35	71.69
13	0	0	+1.68	206.37	71.26
14	0	0	0	252.30	71.00
15	0	+1.68	0	512.30	70.40
16	0	0	-1.68	632.08	85.13
17	0	0	0	247.16	72.00

The size of the microparticles is directly related to the concentration and viscosity of the alginate solution, as low concentrations lead to the reduction of particle size (Sacchetin et al, 2010). In this study, the concentration of 1.16% alginate exhibited greater particle diameters when compared to the concentration of 1.5%, corroborating the findings of Chan (2011) who found greater average diameters, irregular microparticles, and low encapsulation yield when alginate concentrations lower than 1.5% were used.

Likewise, Shi et al. (2013) encapsulated *Lactobacillus bulgaricus* and found an increase in average diameter by increasing the sodium alginate concentration above 1.5%, which affect not only the size but also the microparticles sphericity and flexibility.

3.2. Effect of rotational speed on the diameter of the microparticles

The average size of the microparticles decreased with increasing rotational speed. Although the increased agitation force can lead to a greater dispersion of the internal phase, promoting a thinner emulsion and avoiding agglomerations (Khalil et al, 2010), this did not occur at maximum speed of 338 ×*g*, once an average particle size greater than 500 µm was observed. In this condition, the particles had a larger diameter in comparison with the other rotational speed values, probably due to high turbulence and a more heterogeneous shearing in the reactor, producing larger microparticles despite the addition of emulsifier. According to

Heidebach, Först & Kulozik (2009) this variation in size distribution can be attributed to the high ratio between the viscosity of the dispersed phase and continuous phase, which prevents the rupture of the droplets in the dispersed phase, and allows the production of large microcapsules.

The diameter distribution of the microparticles has been reported by many authors, but few studies have described the optimal particle size. For application in food, the sensory factor is the most important, evidencing an optimum range between 100 – 200 µm (Nag et al., 2011). In this study, microparticles were below 200 µm at rotational speeds between 84.5 and 190 ×*g*. Similar results were observed by Yuan et al. (2008), who produced microparticles by emulsification and found that the rotational speed of around 14.6 - 115 ×*g* provided smaller particle diameters. Khalid et al. (2013) encapsulated oil-in-water emulsion containing L-ascorbic acid, and found that the lower the rotational speed, the more incomplete the homogenization and phase separation is, confirming the present results with the best rotational speeds greater than 58.6 ×*g*.

3.3. Effect of emulsifier concentration on the diameter of the microparticles

The size of the microcapsules can be controlled by the emulsifier concentration, which can form fine droplets, reducing collisions of small droplets and hence decreasing the size of microcapsules (Yuan et al., 2008). In this research, higher emulsifier concentrations led to a decrease in microparticles diameter, and the concentration of 1.5% emulsifier formed particles with diameters in the range 50-200 µm. The results corroborate the findings of Lee et al. (2013), who encapsulated groundnut bud extract by a double water-in-oil-in-water emulsion, demonstrating that the high concentration of hydrophilic emulsifier in the outer oil phase is effective to provide a stable microcapsule. Porta et al. (2012) encapsulated *Lactobacillus acidophilus* by double emulsion, and found that the lower emulsifier concentration (0.4%) did not promote encapsulation of the bacteria, and a better condition for microencapsulation of these cultures was observed with the use of a higher emulsifier concentration (0.8%).

3.4. Process optimization

The particle diameter was affected by all factors, except the interaction between rotational speed (L) and emulsifier concentration (L), which showed a lower statistical significance.

This result is due to the sodium alginate concentration and its interactions are the most important parameters in the formation of microparticles, since they are directly attached to the particle structure. At the same time, the interaction between the two parameters rotation (L) and emulsifier (L) had a less pronounced effect, once these variables directly affect the emulsion (Poncelet et al., 1999).

Eq. (2) represents the statistical model for the variable response diameter of microcapsules:

$$Y = 211.4 + 87.9\ X_1 - 0.8X_1^2 - 9.8\ X_2 + 0.12X_2^2 - 72.65$$
$$X_3 + 0.7\ X_3^2 - 13.5\ X_1X_2 - 10.73\ X_1X_3 + 0.7\ X_2X_3 \quad (2)$$

The contour curves (Fig. 1) showed the changes in diameter as function of the factors studied. The analysis indicates that the decrease in microparticles diameter is in an alginate concentration of 1.5% to 2%. The best rotational speed was between 84.5 and 190 ×*g*. With respect to emulsifier concentration, the most suitable range was from 1.0% for 1.5%. Thus, the decrease in diameter of the microparticle is obtained not only by the use of high rotational values and emulsifier concentration, but also by moderate concentrations of sodium alginate.
The model was validated by F distribution, which suggested that the statistical model explained a maximum of 75% variation. This limited prediction capacity is related to small variation range of the factors studied, as a primary requirement for the production of microparticles of small diameter.

The size and shape of the particles are two important parameters that affect the physical properties of the microcapsule. The particle size increase with the concentration of sodium alginate. Moghaddam et al. (2015) reported that alginate concentrations above 2% (w/v) formed polydisperse microparticles with large diameter. We did not increase the alginate concentrations, since in addition to the alginate has a high viscosity at high concentrations and the difficulty of handling at room temperatures, the appropriate size of the particle for use in foods is between 100 – 200 µm which can not be achieved with high concentrations of the encapsulating agent (Lawless & Heymann, 2010; Liu et al., 2002).

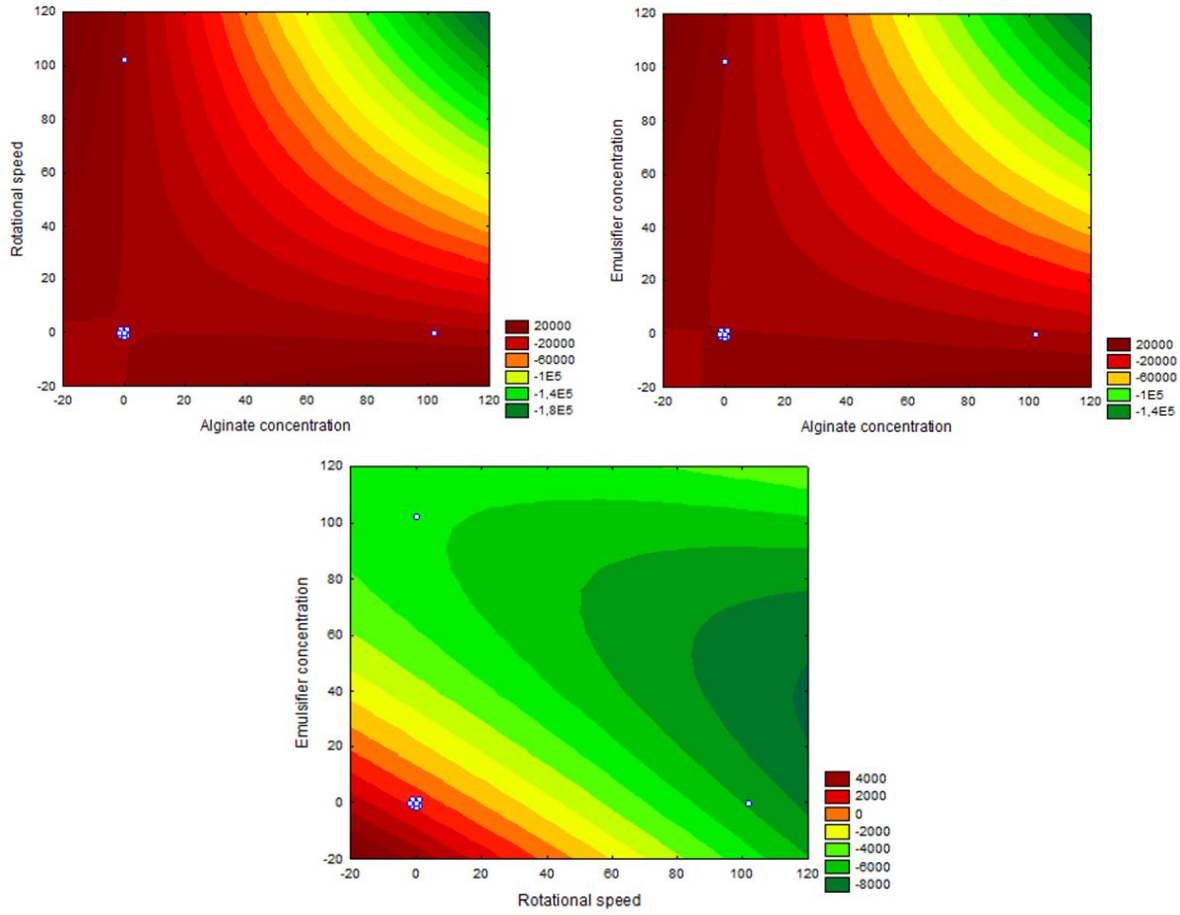

Fig. 1. Contour curves for the variable diameter.

The addition of high concentrations of emulsifying agents tend to decrease the surface tension of the continuous phase, promoting the disaggregation of the particles in the dispersed phase reducing its particle size (Li, Rouaud & Poncelet, 2008). Regarding the concentration of emulsifier, we could not use lower concentrations because the emulsion would not stabilize. Similar to the study of Fei et al. (2015), who demonstrated that the diameter of the microparticles decreased with increasing emulsifier concentration.

With respect to the rotational speed, the average size of the microcapsules decreases with increasing speed. This increase in stirring force leads to a greater dispersion of the internal phase, promoting a finer emulsion and prevent agglomeration. The study of Rosas-Flores et al. (2013), who obtained better results with the rotational speed above 150 ×*g*. So, it used the speed of 84.5 ×*g* as the central point for this research.

3.5. Encapsulation yield

The sodium alginate microcapsules did not significantly affect the encapsulation yield (EY%) for viable cells of *Bifidobacterium* BB-12, which ranged from 65.50% to 99.85%. The encapsulation yield in this study was equivalent to the emulsification/external gelation technique, with EY% values in the range of 80-98% (Song et al., 2013). The maintenance of microoganisms viability is fundamental for the production of microcapsules. Although the most damaging factor in cell viability is the acidic environment, in this study the encapsulation yield was high when compared with previous studies that have reported a lower yield of 50% (Cai et al., 2014; Zou et al., 2011). This is probably due to the acetic acid concentration in the present study was suitable not only to provide the sufficient hydrogenionic potential to initiate gelation, but also to reduce the damage to the organism, since the final emulsion pH was between 4.5 and 5.0, which are still considered tolerance levels for *Bifidobacterium animalis* subsp. *lactis* (Sun et al., 2015). An important factor with respect to the probiotic survival in acidic conditions is that the *B. animalis* subsp. *lactis* culture is more technologically viable than other bifidobacteria species. The acid tolerance of *B. animalis* BB-12 has been associated with the induction of H^+-ATPase because of high survival of this organism under pH values between 3 and 5 for 3 hours (Mättö et al., 2006).

In addition, temperature may be also responsible for the high probiotic survival, once the emulsification process was carried out at room temperature (cold production). In contrast, Anekella & Orsat (2013) used high temperatures (100 °C – 130 °C) for encapsulation of *L. acidophilus* and *L. rhamnosus* in raspberry juice by spray drying, and found lower survival rate of microorganisms from 20% to 50% as a result of thermal shock. The other factors had no significant effect on EY%. Several studies indicate that the encapsulation yield is higher in the emulsification / internal gelation technique. The probiotic yeast *S. boulardii* was encapsulated in calcium alginate microcapsules obtained by emulsification method using inulin and mucilage as coating materials. The encapsulated cells showed higher encapsulation yield of 70% for 35 days of storage (Zamora-Vega et al., 2012). Özer et al. 2008 encapsulated probiotic cultures of *B. bifidum* and *L. acidophilus* by extrusion and emulsification, and reported high viability during the 90 days of storage. Takei et al. 2008 encapsulated *S. cerevisiae* by emulsification, and found encapsulation yield greater than 50% by decreasing the weight ratio of aqueous phase and oil phase.

The results indicate that the encapsulation yield was higher than 65% in all tests. The region considered optimal for the encapsulation process showed yield above 99%. These

results are in agreement with Mokarram et al. (2009) that encapsulated *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* using sodium alginate as coating material. The authors found an average of 99.8% encapsulation yield by emulsification/internal gelation technique, and little reduction in viability after exposure to gastrointestinal conditions. However, other techniques have lower encapsulation yield results due to temperature, pH, and porosity of the microparticle among other factors. In the study conducted by Annan et al. (2008) culture of *Bifidobacterium adolescentis* was encapsulated by extrusion and the results pointed to efficiency lower than 30% in microparticles of gelatin coated with sodium alginate. Hernández-Rodríguez et al. (2014) encapsulated *Lactobacillus plantarum* in whey protein isolate and carrageenan by complex coacervation and found a low encapsulation yield of 39.6%, far below the results obtained in this study.

4. Conclusion

The sodium alginate levels, rotational speed, and emulsifier concentration were decisive in controlling the particle diameter in the microencapsulation of *Bifidobacterium animalis* subsp. *lactis* BB-12.

For the operating conditions using 1.5% sodium alginate, 190 ×*g* rotational speed, and 1.5% emulsifier, the microcapsules showed adequate size distribution with an average diameter of 55 µm and an encapsulation yield greater than 90% with viability of probiotic culture (13.98 CFU g^{-1}). Thus, the study showed an appropriate setting for this process, since a great viability was observed for the microparticles with smaller diameters, facilitating and enabling the application in various food matrices.

5. References

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6. Supplementary material

Table 2
Coefficients of the model estimated by linear regression for diameter

Factor	Effects	Standard error	*t*(2)	*p*	coefficients	Estimates per interval	
						-95%	+95%
Mean	211.44	0.70	297.98	0.00001	211.44	208.39	214.50
X1 (L)	175.82	1.57	111.78	0.00008	87.91	84.52	91.29
X1(Q)	-1.59	0.01	-103.31	0.00009	-0.79	-0.83	-0.76
X2 (L)	-19.60	1.57	-12.46	0.00637	-9.80	-13.18	-6.42
X2(Q)	0.25	0.01	16.19	0.00379	0.12	0.09	0.15
X3 (L)	-145.31	1.57	-92.38	0.00011	-72.65	-76.04	-69.27
X3(Q)	1.42	0.01	92.15	0.00011	0.71	0.67	0.74
X1 (L) × X2 (L)	-27.03	1.81	-14.87	0.00449	-13.51	-17.42	-9.60
X1 (L) × X3 (L)	-21.47	1.81	-11.81	0.00708	-10.73	-14.64	-6.82
X2 (L) × X3 (L)	1.42	1.81	0.78	0.51639	0.71	-3.19	4.61

Table 3
Analysis of variance (ANOVA) for the quadratic model for diameter

Source of variation	Sum of squares	Degrees of freedom	Mean squared	$F_{calculated}$
Regression	623491.1	9	69276.78	3.14[a]
Residues	154184.8	7	22026.40	
Lack of fit	154171.6	5	4668.19	
Pure error	13.2	2	6.61	
Total	777675.9	16		

[a] Statistical significance ($p<0.25$).

5 MANUSCRITO II

Microencapsulation of the probiotics *Bifidobacterium* BB-12 by internal gelation: viability in simulated gastrointestinal conditions and during storage[*]

ABSTRACT

Emulsification/internal gelation is an encapsulation technique with great potential for protection of probiotics for use in food products. In this study, microparticles containing *Bifidobacterium* BB-12 produced by emulsification using sodium alginate as wall material with a slow release of calcium ions were evaluated. Probiotics survival was studied for resistance to simulated gastrointestinal conditions and stability during 120 days of storage. The characterization of the microparticles and stability in buffer solutions at different pH (4.5, 6.0, and 7.5) was investigated. Although the free cells presented low resistance, the encapsulated bacteria were resistant to the simulated gastrointestinal conditions, providing protection to *Bifidobacterium* BB-12. A loss of viability was observed for the encapsulated microorganisms at 7 °C and 25 °C after 120 days, while the best viability was obtained at frozen storage (-18 °C), with a counts of 7.31 log CFU g^{-1} at the end of storage. The microparticles presented stability in buffer pH 4.5 and total release of probiotics at pH 7.5. The study indicates that the emulsification / internal gelation has proven to be a viable technology for protection, application, and controlled release of probiotics.

Keywords: probiotics; microencapsulation; sodium alginate; internal ionic gelation; *Bifidobacterium*

[*] Manuscrito aceito para a publicação na Revista Internacional *LWT- Food Science and Technology*.

1. Introduction

Bifidobacteria are non-sporulating species, anaerobic, gram-positive rods and catalase negative. They can grow at temperatures from 25 to 45 °C and pH 4.5 to 8.5, with optimal range at pH 6.5 to 7 (Fellows, 1992; Scheinbach, 1998). They are considered one of the most important groups of probiotic bacteria and have been mainly incorporated into fermented foods and dairy products, due to their numerous beneficial effects as maintenance of gut microflora and stimulation of the immune system, among others (Modler & Villa-Garcia, 1993; Patel et al., 2015).

However, for exerting health benefits, probiotics should be able to tolerate the acidic conditions of the stomach and the small intestine bile. Stomach acid environment and bile salts secreted into the duodenum are the main obstacles to probiotic bacteria (Anal and Singh, 2007; Castro-Cislaghi et al, 2012.). The survival of *Bifidobacteria* in the gastric pH is considered low, and several factors have been reported to significantly affect the viability of probiotics in foods such as acidity, pH, dissolved oxygen levels, and storage time and temperature (Prasanna, Grandison & Charalampopoulos, 2014; Pinto et al., 2015). Thus, a major challenge faced in developing a probiotic product is to ensure a high bacteria survival rate during manufacturing and shelf life of food products so that these microorganisms are delivered in sufficient amounts to confer the expected benefits (Tripathi & Giri, 2014; Champagne et al., 2015).

In this context, the microencapsulation has been proposed as an alternative for increasing the survival of probiotic bacteria in food processing and storage as well as during the passage through the human gastrointestinal tract. Various methods for probiotics microencapsulation with alginate have been reported, including spray-drying, spray-coating, extrusion, emulsification, and complex coacervation (Champanhe & Fustier, 2007; Martín et al., 2015).

The emulsification technique has been widely used for microencapsulation due to its low cost, mild formulation conditions, and high cellular retention (Takei, Yoshida, Hathath, Shiomori, & Kiyoyama, 2009). In the emulsification / internal gelation, an aqueous polysaccharide solution is dispersed into the oil phase to form a W / O emulsion, followed by addition of calcium chloride solution to the emulsion while stirring, thus occurring the encapsulation of probiotics (Mokarram et al., 2009). Studies have shown that the emulsification technique with sodium alginate has great potential for application to probiotic

cultures, since it increases the viability of probiotic bacteria around 80% (Le-Tien, Millette, Mateescu & Lacroix, 2004; Goh et al., 2012; Amine et al., 2014).

Therefore, the aim of this study was to evaluate the stability of microparticles containing *Bifidobacterium* BB-12 produced by emulsification / internal gelation using sodium alginate as wall material under simulated gastrointestinal conditions, as well as the probiotic viability during storage.

2. Material and Methods

2.1. Materials

Sodium alginate was kindly provided by FMC BioPolymer (Campinas, Brazil). Canola oil (Liza®, Cargill, Mairinque, São Paulo, Brazil). $CaCO_3$ (calcium carbonate) was acquired by Neon Comercial Ltda (São Paulo, Brazil). Other products such as Tween 80 (Polysorbate 80), $C_2H_4O_2$ (Glacial Acetic Acid), and $CaCl_2.2H_2O$ (Calcium Chloride Dihydrate) were acquired by Vetec Química Fina Ltda (Rio de Janeiro, Brazil).

2.2. Cultures

The lyophilized culture of *Bifidobacterium animalis* subsp. *lactis* BB-12 was kindly supplied by Chr. Hansen (Valinhos, Sao Paulo, Brazil) with cell viability of about 14 log CFU g^{-1}. This culture was stored at -18 °C until the time of use.

2.3. Microencapsulation process

The microparticles were produced by the emulsification / internal gelation technique according to Poncelet (1992) with modifications. For that, 40 mL sodium alginate solution (1.5%), 1g freeze-dried culture and 2 mL $CaCO_3$ suspension (500 mM Ca^{2+}) were mixed. The mixture was transferred to a round-bottom reactor (60 mm internal diameter and 90 mm height) and dispersed into 200 mL canola oil containing 1.5% Tween 80, at 900 rpm for 15 minutes, using an automatic stirrer Macro Quimis® (Diadema, Sao Paulo, Brazil), provided with a three-blade marine type propeller (8 mm thick and 42 mm internal diameter between the blades), forming a water in oil emulsion. Then, 40 mL canola oil containing 160 µL of glacial acetic acid were added to the emulsion and stirred for 5 minutes. The beads were

maintained in 0.05M $CaCl_2$-$2H_2O$ and after complete migration to the aqueous phase, the oil was discarded and the microcapsules were filtered in 0.038 mm (400 mesh) sieve and washed in distilled water at pH 4.0 until complete oil removal, and stored at 4 °C for further analysis

2.4. Enumeration of microencapsulated Bifidobacterium BB-12

Bacteria release from the microcapsules was performed according to the method of Sheu & Marshall (1993) with modifications. For that, 1 g of microparticles was suspended in 9 mL phosphate buffer (0.1 M, pH 7.5), homogenized in a stomacher for 7 minutes, and the breaking of the capsules was confirmed by optical microscopy. The enumeration of *Bifidobacteria* was carried out by serially dilutions with peptone water (0.1 g 100 g^{-1}), plated on modified MRS agar containing lithium chloride (0.1%) and L-cysteine (0.05%), according to manufacturer recommendations (Chr Hansen, 1999). Plates were incubated under anaerobic conditions in anaerobic jars with Anaerobac system (Probac, Sao Paulo, Brazil) at 37 °C for 72 h. After the incubation period, the probiotic viable cell was enumerated and the results were expressed as log colony forming units per gram (CFU g^{-1} log).

2.5. Characterization of the microparticles

2.5.1. Morphology and particle size distribution of the microparticles

The morphology of the microparticles was evaluated using an optical microscope (Carl Zeiss Axio Scope.A1, Oberkochen, Germany) equipped with a digital camera Axio Cam MRc (Carl Zeiss). The particle size distribution was determined in the Mastersizer 2000 equipment (Malvern, Germany) using water as the dispersion medium.

2.5.2. Physicochemical characterization

The microparticles were characterized for moisture (% w / w), lipids (% w / w) and ash (% w / w) according to AOAC methodologies (2005). The carbohydrate content was calculated by the difference between 100 and the sum of the proteins, lipids, ash, and moisture. All analyses were performed in triplicate and the results expressed as a percentage.

2.5.3. Color measurements

The color of the microcapsules was measured using a colorimeter (MinoltaChroma Meter CR-400, Osaka, Japan), previously calibrated and adjusted to operate with illuminant D65 and 10 ° angle. The CIELab color scale was used to measure the parameters L *, a * and b *, where L * represents brightness from black (0) to white (100), a * represents variation from green (-) to red (+), and * b represents variation from blue (-) to yellow (+).

2.6. Stability of the microparticles and in vitro release of bifidobacteria

The microparticles were added with probiotic initial count of 7.74 log CFU g^{-1} to phosphate buffer solutions at pH 7.5 and pH 6.0, and to acetate buffer pH 4.5, and agitated at 150 rpm and 37 °C in a refrigerated incubator shaker (TE-421, Tecnal, Piracicaba, SP, Brazil). Aliquots were sampled after 60, 120, and 180 minutes for enumeration of *Bifidobacterium* BB-12 released from the microparticles. The integrity of the particles was monitored by light microscopy before and after the stability test, concomitant with the microbiological analyses.

2.7. Survival of free and microencapsulated Bifidobacterium BB-12 after exposure to simulated gastrointestinal conditions

The simulation of gastrointestinal conditions was performed according to the method proposed by Madureira et al. (2011) with modifications. The viability of bacteria exposure to sequential simulated gastro and intestinal condition was assessed (esophagus / stomach, duodenum and ileum). To simulate the passage through the esophagus / stomach, 1 g of microcapsules or 1 g of lyophilized culture was homogenized with 25 mg mL^{-1} of pepsin (Sigma, Germany) in 0.1 M HCl at a concentration of 0.05 $mL.mL^{-1}$ for 90 minutes, and the pH adjusted to 2.0 with 1M HCl. To simulate the passage through the duodenum, a solution containing 2 g L^{-1} pancreatin (Sigma) and 12 $g.L^{-1}$ of bovine bile salts (Sigma) in 0.1M $NaHCO_3$ at pH 5.0 was used at a concentration of 0.25 mL^{-1} mL. Finally, for the simulated passage through the ileum, the pH was adjusted to 6.5 using 0.1 M $NaHCO_3$. All solutions were prepared on the day of analysis and sterilized by membrane filtration with 0.20 µm pore (Millipore, Billerica, MA, USA). The analysis was performed in a refrigerated incubator shaker (TE-421, Tecnal, Piracicaba, SP, Brazil) at 37 °C to simulate the body temperature at

agitation conditions (130 rpm for esophageal / stomach assay, and 45 rpm for duodenum and ileum assay) to simulate the peristaltic movements of the digestive tract. During the procedure, aliquots were taken after 0 min, 90 min (esophageal / stomach), 110 min (duodenum), and 200 min (ileum), and the survival of free and microencapsulated *Bifidobacterium* BB-12 was analyzed by enumeration in MRS agar as performed for enumeration of the microencapsulated *Bifidobacteria* (Section 2.4).

2.8. Viability of Bifidobacterium BB-12 at different storage temperatures

The viability of the microencapsulated bacteria during storage was investigated to determine the shelf life of the microparticles. The viable cells were enumerated after 0, 15, 30, 45, 60, 75, 90, 105, and 120 days of storage at -18 °C, 7 °C and 25 °C using MRS agar as described in Section 2.4.

2.9. Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using Statistica Software version 7.0 (2004) (Statsoft Inc., Tulsa, OK, USA), followed by the Tukey test to compare means, at a 5% significance level, if there are significant differences between treatments. All tests were performed in triplicate and data were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Caracterization of the microparticles

The external morphology and internal structure of the microcapsules, and the particle size distribution of the filling material (microorganisms) within the matrix was assessed by optical microscopy (Fig. 1). In general, the microcapsules were spherical, with the filling material distributed throughout the matrix. Sodium alginate (encapsulating material) and microorganisms (active material) were found throughout the interior of the microparticle, which characterized it as a matrix type, once the active material is not only located in the core, but inside the particle (Jafari, Assadpoor & Bhandari, 2008).

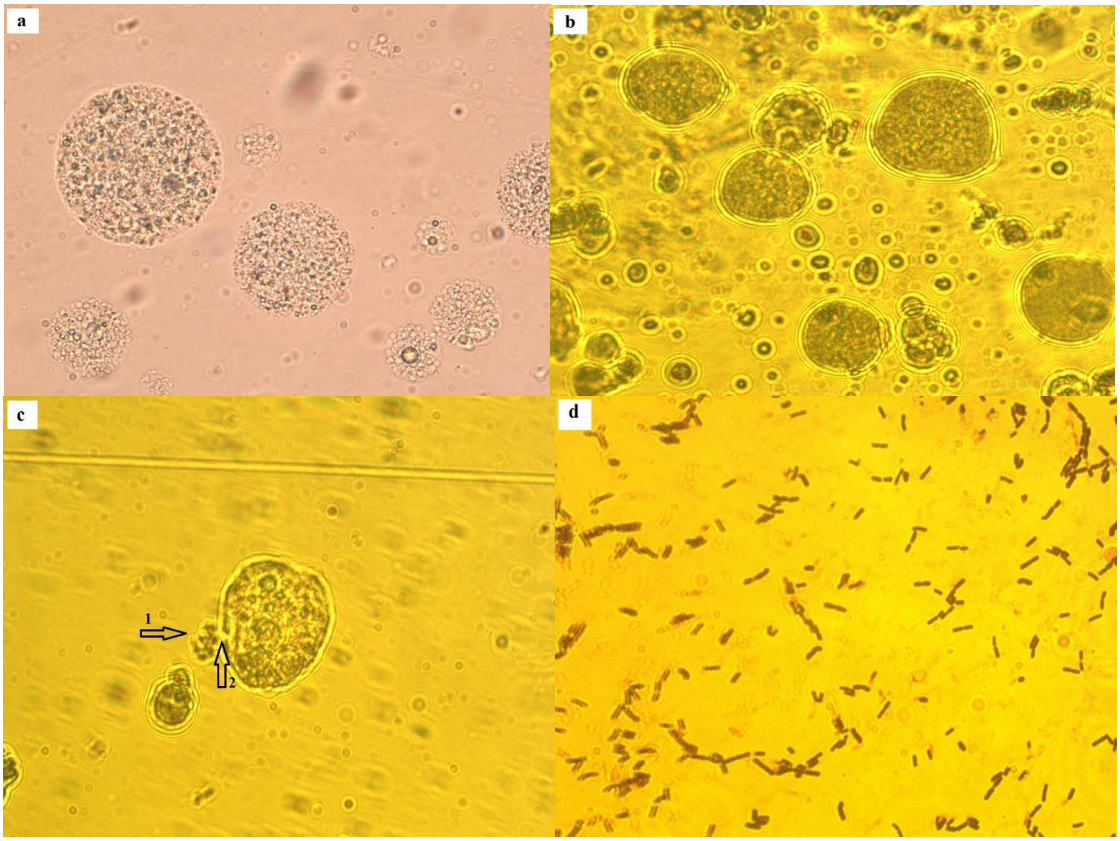

Fig. 1. Optical microscopy microparticles of *Bifidobacterium* BB-12. (a) Alginate microparticles containing *Bifidobacterium* BB-12 (40×). (b) Microparticles with the presence of oil (40×). (c) Alginate microparticle, where the number 1 is showing the free microorganism of the microparticle and the number 2 is showing a break in the microcapsule in the buffer solution 7.5 (40×). (d) Free probiotics (100×).

No aggregation was observed due to the oil concentration on the surface. Ribeiro et al. (2005) produced alginate-chitosan micro particles by emulsification and observed particle aggregation due to the strong electrostatic interaction between the two polysaccharides oppositely charged, which was not observed when the microparticles were coated with only one of the polysaccharides.

The microparticles had an average diameter of 54.82 µm (Table 1), corroborating the study by Zou et al. (2011), who reported that the diameter of the microparticles obtained by emulsification / internal gelation can vary from 50 - 120 µm. Similar results were obtained by Song et al. (2013), who studied microencapsulation of probiotics (yeast, Y235) by emulsification / internal gelation and found particles with 35-350 µm, with more spherical shape, and smooth and uniform surface. Several studies have reported that the size distribution of the microparticles may affect both the encapsulation efficiency and the texture of the products in which they are incorporated. Diameters smaller than 100 µm are preferred for most applications due to better protection against the gastric juice (Champagne & Fustier,

2007; Arup et al, 2011). The average diameter of the microparticles produced in this study is within this limit.

Table 1
Characteristics of microparticles of *Bifidobacterium* BB-12 obtained by internal ionic gelation with alginate.

	Microparticles with alginate*
Particles size (µm)	54.82 ± 0.54
Moisture (%)	96.15 ± 0.12
Fat (%)	2.23 ± 0.36
Carbohydrates (%)	1.08 ± 0.29
Ash (%)	0.49 ± 0.06
Colour attributes	
L*	47.67 ± 1.71
a*	-1.90 ± 1.33
b*	13.22 ± 1.74

* Results expressed as mean ± standard deviation.

Regarding the color attributes, the parameter L* (luminosity) was slightly higher (Table 1), indicating clear color of the microparticles. Low a* and high b* values were also observed, indicating the capsules tended to yellow and green, respectively. The canola oil interfered with the color of the microcapsules due to its yellow-green color, as well as sodium alginate that is white -yellow, and may also have contributed to the intensity of yellow color of the microparticles. According to Aryana & McGrew (2007), the color of the ingredients used in the formulations greatly affects the color of the final product. This explains the tendency of the microparticles to yellow, since both oil and alginate contribute to the final color. Studies have shown loss of viability of probiotic bacteria during cheese manufacture, thus the incorporation of these microparticles into a product with similar color may be a promising alternative (Ortakci, Broadbent, McManus, & McMahon, 2012; Rosas-Flores et al., 2013).

Table 1 shows the physicochemical characterization of the microparticles. High moisture levels (mean 96.17% ± 1.82) were observed, corroborating the results found by Mukai-Correa et al. (2005), who produced microcapsules by internal gelation containing casein and hydrogenated vegetable fat, and found moisture levels greater than 90%, due to high water retention capacity of polysaccharide-based microparticles.

Lipids levels were around 2.24%, probably due to the small amount of oil on the surface of the capsule. The microcapsules presented 0.56% ash and 1.08% carbohydrates, due to the insoluble calcium at the beginning of the process and sodium alginate used as wall

material, respectively. Ribeiro et al. (2014) studied *Lactobacillus acidophilus* LA-5 microencapsulated by internal gelation and complex coacervation in yogurts, and reported that although the microparticles did not affect the physicochemical characteristics of the product, changes were observed when free probiotics were used. Similar results were observed by Homayouni et al. (2008), who studied *Lactobacillus casei* and *Bifidobacterium lactis* microencapsulated by emulsification in ice cream, and concluded that the encapsulation significantly improved the probiotics survival, with no significant changes in the physicochemical composition of the product.

3.2. Stability of the microparticles and in vitro release of Bifidobacteria

The release of *Bifidobacterium* BB-12 from the microparticles after the stability tests at different pH values is shown in Table 2. Lower counts were observed at pH 4.5, ranging from 3.12 to 3.79 g^{-1} log CFU for all periods studied. This result shows that there was practically no release or dissolution of the particles in this environment, and the lower counts were probably due to the cells on the surface and those detaching from the particles. At pH 6.0, intermediate counts were observed, remaining between 4.50 and 6.8 log CFU g^{-1}. Probably, the dissolution of the particles was not complete in this environment. Finally, at pH 7.5, higher counts were observed, reaching 6.60 log CFU g^{-1} (Fig. 1c and 1d).

Table 2
Populations of *Bifidobacterium* BB-12 in the microparticles obtained by internal ionic gelation and treated with solutions pH 4.5, 6.0 and 7.5

Time (minutes)	pH		
	4.5	6.0	7.5
60	$3.12^{aA} \pm 0.04$	$4.50^{aB} \pm 0.06$	$4.72^{aC} \pm 0.05$
120	$3.39^{bA} \pm 0.04$	$5.32^{bB} \pm 0.04$	$5.10^{bC} \pm 0.01$
180	$3.79^{cA} \pm 0.05$	$6.08^{cB} \pm 0.07$	$6.60^{cC} \pm 0.05$

$^{a-c}$ Within a column, different superscript lowercase letters denote significant differences ($p < 0.05$) among the different incubation times in the same pH.
$^{A-C}$ Within a line, different superscript uppercase letters denote significant differences ($p < 0.05$) among the different pH values in the same incubation time.

It is worth emphasizing the different release rates observed at pH 4.5, 6.0, and 7.5, once significant differences ($p < 0.05$) were observed for the probiotic populations as a function of the release time. This release profile shows that these particles have potential for application in foods with pH below 4.5, with possible controlled release in the intestine.

Similar results were observed by Déat-Lainé et al., (2012), who investigated microencapsulation of insulin with alginate and protein by extrusion, and found a gradual release of the bioactive compound in acidic pH, whereas a sudden release was observed in alkaline pH due to polymers degradation. In another study, Park et al., 2014 investigated the microencapsulation of astaxanthin by extrusion and observed that the alginate is converted into insoluble alginic acid under acidic conditions, preventing the diffusion of the bioactive compound, while in alkaline environment the alginate dissolution led to a rupture of the microparticles.

3.3. *Survival of free and microencapsulated Bifidobacterium BB-12 exposed to simulated gastrointestinal conditions*

Fig. 2 shows the survival of *Bifidobacterium* BB-12 after exposure to simulated gastrointestinal conditions, for both free and microencapsulated cells with sodium alginate by emulsification / internal gelation. After 90 min at pH 2.0 (simulating esophageal / stomach environment) a reduction of 3.04 log CFU / g ($p < 0.05$) in free cells was observed when compared with the initial bacteria counts. Our results demonstrated that the exposure of free cells to acidic conditions and gastric enzymes resulted in a decreased viability of the probiotic bacteria. These findings corroborate Bove et al. (2013), who reported a higher stress when *Lactobacillus plantarum* was subjected to simulated gastric conditions. Ranadheera et al. (2012) studied the viability of probiotics in ice cream and goat milk yoghurt, and reported a greater inactivation of probiotic bacteria when exposed to acid pH values (~pH 2) when compared to basic pH.

When the microencapsulated cells were subjected to acidic conditions (simulating esophageal / stomach environment), a reduction of 9.10 log CFU g^{-1} ($p < 0.05$) was observed when compared the initial and final bacteria counts, which were 13.42 and 4.32 g^{-1} log CFU, respectively. However, this lower count is not due to the low viability as occurred in free cells, since no particle disruption and thus no bacteria release was observed at pH 2. The present results corroborate with the findings of Cai et al. (2014), who studied the microencapsulation of *Lactobacillus acidophilus* by emulsification with alginate, and observed survival of the microorganisms under acidic conditions, probably due to better mechanical properties and density of the microparticles. Other authors have reported that the microencapsulation with sodium alginate is effective for the survival of probiotics in acidic conditions (Doleyres & Lacroix, 2004; Ding & Shah, 2007).

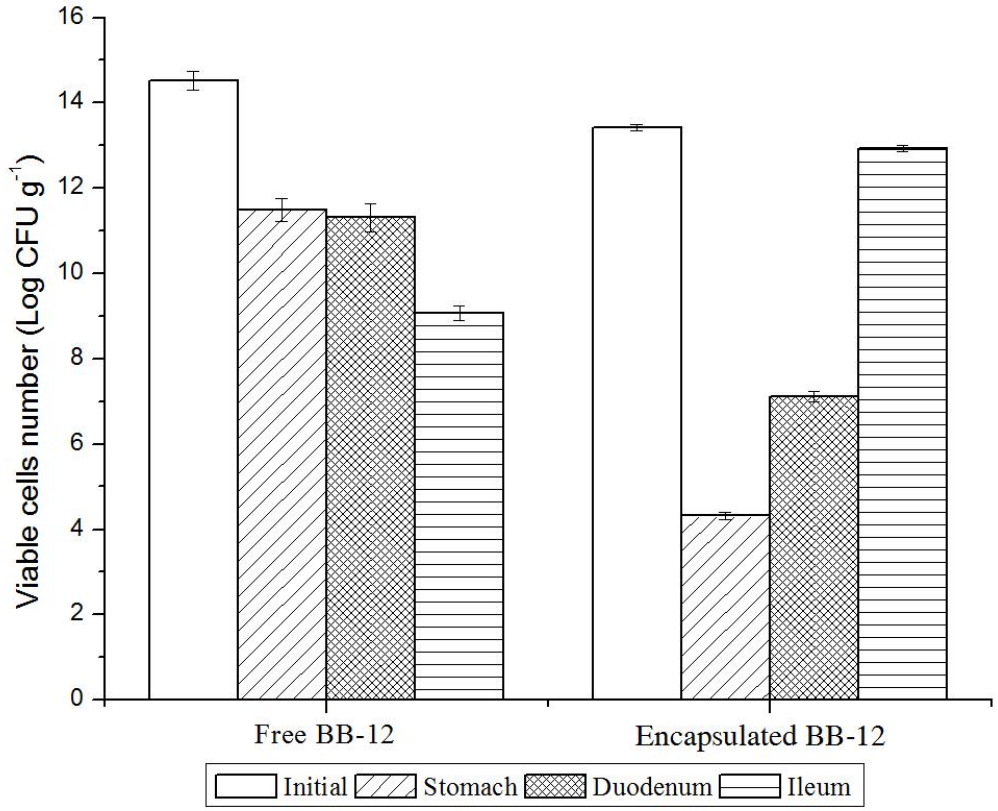

Fig. 2. Survival of *Bifidobacterium* BB-12 free and encapsulated after each step of the simulated gastrointestinal conditions.

As can be seen in Fig. 2, *Bifidobacteria* viable cells count in the microcapsules after exposure to simulated duodenum conditions (i.e., bile salts, pancreatin, and pH 5.0) was 7.12 g^{-1} log CFU ($p < 0.05$), suggesting particle disruption, which led to higher cell counts as compared to acidic conditions. In contrast, no significant difference was observed for the free cells, with a small decrease of 0.17 log CFU g^{-1} ($p > 0.05$) due to cell damage caused by acidic conditions. Thus, increasing the pH in the duodenum was favorable to probiotic survival in the microparticles. Maciel et al. (2014) have also reported an increase in the viability of *L. acidophilus* La-5 microencapsulated with sweet whey or skimmed milk by spray-drying during exposure to simulated gastrointestinal conditions, at pH 2 to 7. Pinto et al. (2015) studied the microencapsulation of *Bifidobacterium* BB-12 with whey by spray drying, and observed a 12% increase in probiotics viability. Similarly, the present study conferred a full protection of probiotics in the alginate microparticles obtained by emulsification.

After exposure to the simulated ileum conditions (pH 6.5), a decrease of 0.49 g^{-1} log CFU was observed for the encapsulated microorganism, when compared with the initial counts ($p < 0.05$), probably because the microencapsulation process conferred protection to

probiotic bacteria against the gastrointestinal simulation, maintaining the viability of the probiotics. With regard to the simulated duodenum condition, a release of 5.81 log CFU g^{-1} ($p < 0.05$) of viable *Bifidobacteria* cells was observed in the simulated ileum section. A disruption of all microparticles was observed in the simulated gastrointestinal tract, releasing *Bifidobacterium* cells in the desired location. These results are in agreement with those obtained by Chandramouli, Kailasapathy, Peiris & Jones (2004), who studied *Lactobacillus acidophilus* encapsulated with sodium alginate using an encapsulating machine®, and found 10^9 CFU g^{-1} after exposure to gastrointestinal conditions. Iyer and Kailasapathy (2005) studied the probiotics *Lactobacillus acidophilus* encapsulated with modified starches subjected to simulated gastrointestinal conditions, and observed higher survival of the encapsulated probiotics when compared to the free cells.

As regards the free cells, a reduction of 5.46 g^{-1} log CFU ($p < 0.05$) was observed when compared with the initial counts, due to pH variation during the simulation of the passage through the gastrointestinal (GI) tract. Bedani, Rossi, Saad (2013) have reported that *Bifidobacterium animalis* BB-12 in a fermented soy product was more tolerant during the simulated passage through the GI tract when compared with *Lactobacillus acidophilus*, with probiotic counts of 8 log CFU g^{-1}, corroborating the current study.

3.4. *Stability of the microencapsulated microorganisms during storage*

Cell damage and loss of activity may occur during processing and storage. Therefore, a suitable microencapsulation process should ensure bacteria survival during the processing steps, which should remain viable throughout the storage period (Oliveira et al, 2007; Champagne et al, 2015). However, a minimum viable probiotics count is required until the time of consumption to confer health benefits. In general, the food industry has adopted the minimum recommended level of 10^6 CFU g^{-1} product (Tripathi & Giri, 2014; Kailasapathy & Rybka, 1997).

In this study, according to the results shown in Fig. 3, the *B. lactis* BB-12 counts decreased during storage, with a reduction of 6.74 log CFU g^{-1} at 25 °C after 120 days of storage. However, the probiotic viability was observed up to 75 days of storage, confirming the results of Alves et al. (2015), who encapsulated *Lactobacillus plantarum* by extrusion using alginate and corn starch as a wall materials, and concluded that the storage at room temperature led to a significant reduction of more than 2 log after 30 days. This is probably

due to a metabolic activity of the microorganisms at 25 °C, resulting in cell death and loss of cell viability (Soto et al., 2011).

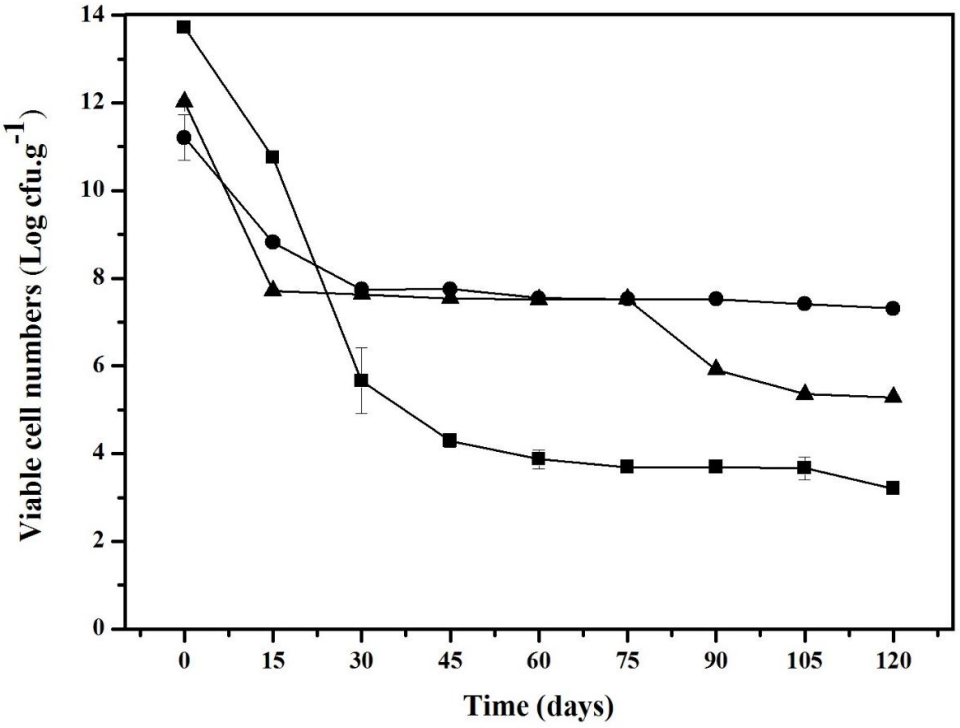

Fig. 3. Viability of *Bifidobacterium* BB-12 microencapsulated during storage at -18 °C (●), 7 °C (■) and 25 °C (▲).

With respect to the refrigerated storage (7 °C), there was a greater reduction in probiotic viable cells (10.52 g^{-1} log CFU) when compared to the other storage conditions. Ding & Shah (2008) have shown that the microencapsulation of different probiotic species by internal gelation, and application of the microparticles in fruit juice led to a decline in viability of free *L. acidophilus* after five weeks under refrigerated storage (7 °C). The present study showed a probiotic viability up to two weeks of storage, with subsequent decline ($<10^6$ CFU g^{-1}), which remained for 120 days. Despite the refrigerated storage is a suitable condition for the retention of microorganisms and stability of the microparticles, a decrease in bacteria viability was observed, probably due to a number of factors, such as oxidation of fatty acids, production of free radicals in the presence of oxygen, and damage to cellular DNA (Castro, Teixeira, & Kirby, 1997; Pedroso, et al., 2012).

After 120 days of frozen storage (-18 °C), a count of 7.31 log CFU g^{-1} was observed for the microencapsulated cells, suggesting that higher stability increases with the decrease in temperature, since an impairment in the rearrangement of crystals and exposure of the active ingredient is observed at lower temperatures, conferring longer shelf life for the

microparticles. Similar results were found by Corcoran et al., (2004), who evaluated the survival of *Lactobacillus rhamnosus* associated with prebiotics, and found a higher survival rate at lower temperature conditions. Martín et al., (2013) studied microencapsulation of *Lactobacillus fermentum* with sodium alginate by emulsification and observed a reduction of viability of only 0.8 log CFU g^{-1} after 45 days of frozen storage (at -20 °C), which remained stable thereafter.

Whereas the microorganisms are metabolically active in the microparticles at 7 °C and 25 °C, this marked decrease can be due to the production of various metabolite compounds, bacteriocins and / or absence of substrates that may lead to the inactivation of the probiotic bacteria (Castro, Teixeira & Kirby, 1997). As in the present study, Pedroso et al. (2012) have produced solid lipid particles with *Bifidocaterium* BB-12 encapsulated by spray-chilling, and found a significant reduction of 7.80 log CFU g^{-1} after 90 days of storage at 37 °C.

4. Conclusion

The results have shown that the technology used in this study is suitable for microencapsulation of *Bifidobacterium* BB-12 using sodium alginate as coating material. The emulsification/internal gelation has provided effective protection of probiotics during the passage through simulated gastrointestinal fluids and buffer solutions at pH 4.5. The technique also conferred probiotic viability during 120 days of frozen storage (-18 °C). Furthermore, the suitable morphology, particle size distribution, and physicochemical composition can provide better incorporation of the microparticles into food products, preventing the negative effects on the sensory properties such as texture and flavor.

Therefore, this novel method is capable of producing stable microparticles for potential application of probiotics in food at low cost and on an industrial scale.

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6 MANUSCRITO III

Development and characterization of alginate microcapsules containing *Bifidobacterium* BB-12 produced by emulsification/internal gelation followed by freeze drying*

ABSTRACT

Microencapsulation is one of the most efficient methods of protecting microorganisms and is a promising technique to increase the viability of probiotic cultures. However many challenges still exist with respect to the adaptation of innovative techniques, the coating materials used and the food matrixes using microencapsulated probiotics. Emulsification/internal gelation is an encapsulation method showing great potential to confer protection on probiotics that need to be added to foods with an unfavorable environment for their viability. The objective of this study was to produce probiotic microcapsules by internal ionic gelation associated with freeze drying, and evaluate the viability of the probiotics under simulated gastrointestinal conditions and their stability during storage under distinct environmental conditions for 120 days. The results showed that microencapsulation resulted in an elevated viability of the probiotics and that the microcapsules were resistant to the simulated gastrointestinal conditions, providing substantial protection to the *Bifidobacterium* BB-12. The encapsulated microorganisms maintained their viability of 7.89 log CFU g^{-1} for up to 60 days at 25ºC, but the best results were maintained when submitted to refrigerated (7ºC) or frozen (-18ºC) storage. In addition the microcapsules remained protected in a solution buffered at pH 4.5 but showed total release of the probiotics in a solution buffered at pH 7.5, showing conditions of controlled release for use with probiotic carriers for human consumption. Thus the study demonstrated that emulsification/internal ionic gelation associated with the freeze drying process could be considered feasible technology for the protection, application and controlled release of probiotics for use in foods.

Keywords: probiotics; microencapsulation; sodium alginate; internal ionic gelation; freeze drying

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1. Introduction

A series of benefits have been attributed to probiotic bacteria, especially those of the genus *Bifidobacterium* which are anaerobic, gram-positive and are normal inhabitants of the human colon. They show numerous positive effects on the health including an improvement in the intestinal flora by preventing the colonization of pathogenic microorganisms, an increase in protein digestion and activation of the immune system. Thus the bifidobacteria have been widely incorporated into food products (Gomi et al., 2015; Lian, Hsiao & Chou, 2003; Verruck et al., 2015).

Although these microorganisms have probiotic properties, offering consumer health benefits, the incorporation and viability of these bacteria in the product still represent a technological challenge for the food industry. Various factors affect the viability of bifidobacteria, including the acidity, pH value, storage temperature and oxygen content. Thus microencapsulation is a viable alternative for the protection of probiotics, conferring greater viability to the strains and greater effectivity of the probiotic action due to controlled release (Anal & Singh, 2007; Ranadheera et al., 2010).

Microencapsulation by emulsification/internal ionic gelation has the property of producing particles formed from a water in oil emulsion. Thus the encapsulating solution must contain a polymer such as sodium alginate, a vegetable oil source, calcium carbonate, a surface active agent and an organic acid which reacts specifically with the calcium carbonate to form the microcapsules. One of the great advantages of this method is the production of small particles (<100 μm), which is very convenient since it avoids the need for specialized equipment and complex techniques, conferring low costs due to the simple formulation conditions with a high yield of cell viability, making this technique one of the most promising ones (Cook et al., 2012; Eng-Seng et al., 2011).

The drying of the microcapsules is an important factor in research with microencapsulation, since the majority of studies have reported using moist microcapsules. There are various advantages to drying the microcapsules including an improvement in the storage properties of the capsules due to the removal of water and the ease in use and homogenous distribution throughout the product (Mortazavian, Razavi, Ehsani, & Sohrabvandi, 2007). Probiotic cell concentrates frequently require long storage periods before the manufacture and consumption of the product. Thus the probiotic microcapsules must be dried after production, this being important especially for dry products, such as beverage powders and cereals, where the microcapsules must be added dry. However the impact of

microencapsulation and drying before their addition to the systems has been little investigated as yet (O'Riordan et al., 2001; Su et al., 2007; Heidebach, Först&Kulozik, 2010; Sousa et al., 2012).

Thus the objective of the present study was to produce probiotic microcapsules by internal ionic gelation associated with freeze drying and evaluate the viability of the probiotics under simulated gastrointestinal conditions and their stability during 120 days of storage under distinct environmental temperature conditions.

2. Material and methods

2.1. Materials

Sodium alginate was kindly provided by FMC BioPolymer (Campinas, Brazil), rapeseed oil by Liza®, Cargill, Mairinque, São Paulo, Brazil, $CaCO_3$ (calcium carbonate) was acquired from Neon Comercial Ltda (São Paulo, Brazil) and other products such as Tween 80 (Polysorbate 80), $C_2H_4O_2$ (glacial acetic acid) and $CaCl_2$. $2H_2O$ (dihydrated calcium chloride) were acquired from Vetec Química Fina Ltda (Rio de Janeiro, Brazil).

2.2. Culture

The freeze dried culture of *Bifidobacterium animalis* subsp. *lactis* BB-12 was kindly donated by Chr. Hansen (Valinhos, São Paulo, Brazil) with a cell viability of approximately 14 log CFU g^{-1}. The culture was stored at -18 ºC until the moment of use.

2.3. Microencapsulation

The microcapsules were produced by the technique of emulsification/internal gelation developed by Poncelet (1992) with modifications. An aliquot of 40 mL of a 1.5% sodium alginate solution was mixed with 1g of the freeze dried microorganism and 2 mL of a $CaCO_3$ suspension (500 mM Ca^{+2} equivalents). This mixture was transferred to a round bottomed reactor (internal diameter of 60 mm and height of 90 mm) and dispersed for 15 minutes in 200 mL of rapeseed oil containing 1.5% Tween 80 at a rotational velocity of 900 rpm, using a Quimis® macro electronic mechanical stirrer (Diadema, São Paulo, Brazil) equipped with a

three bladed marine helix (8 mm thick and 42 mm internal diameter between the blades) so as to form a uniform water in oil emulsion. While stirring continuously, 40 mL of rapeseed oil containing 160 µL glacial acetic acid, were added to the emulsion. After 5 minutes the stirring was stopped and the capsules were separated by adding 300 mL of a 0.05 M dihydrated calcium chloride solution. After the complete separation of the microcapsules into the aqueous phase, the oil was discarded and the capsules filtered through sieves with a porosity of 0.038 mm (400 mesh) and washed with distilled water at pH 4.0 until the complete removal of the oil.

2.4. Freeze drying

The microcapsules produced were frozen (-18ºC for 24 hours) on the same day they were produced. The frozen microcapsules (-18ºC) were freeze dried in a Liotop L101 freeze dryer (São Carlos, São Paulo, Brazil) and removed from the freeze dryer 24 h later (vacuum: 0.200 – 0.300 µHg and condenser temperature of -37ºC).

2.5. Bifidobacterium BB-12 count in the microcapsules

The microencapsulated bacteria were released from the capsules according to the method of Sheu & Marshall (1993) with modifications. One gram of microcapsules was suspended in 9 mL of phosphate buffer (0.1 M, pH 7.5) followed by homogenization in a stomacher for 7 minutes. Release of the probiotic cultures was confirmed by verifying rupture of the capsules by optical microscopy. To enumerate the bifidobacteria, serial dilutions were prepared in peptone water (0.1 g 100 g^{-1}) and seeded in MRS agar modified by the addition of lithium chloride (0.1%) and L-cysteine (0.05%) according to Chr Hansen (1999). The plates were incubated in anaerobic jars using the Anaerobac system (Probac, São Paulo, Brazil) to generate anaerobiosis at 37ºC for 72 h. After the incubation period the viable probiotic cells were counted and expressed in log colony forming units per gram (log CFU g^{-1}).

2.6. Encapsulation efficiency

The encapsulation efficiency (%EE), which is the survival rate of the microorganisms during the microencapsulation process, was calculated according to Eq. (1) as proposed by Martin et al. (2013).

$$\%EE = (N/N_0) \times 100 \quad (1)$$

Where N is the number of viable cells (log CFU g^{-1}) released from the microcapsules and N_0 is the number of viable cells (log CFU g^{-1}) in the cell concentrate used for microencapsulation.

2.7. Characterization of the microcapsules

2.7.1. Microcapsule morphology and particle size

The morphology of the microcapsules was evaluated using an optical microscope (Carl Zeiss Axio Scope.A1, Oberkochen, Germany) equipped with an Axio Cam MRc digital camera (Carl Zeiss) and scanning electronic microscope (SEM) (JEOL JM6360, Tokyo, Japan). The particle size distribution of the microcapsules was measured using a Mastersizer 2000 (Malvern, Germany) using water as the dispersion medium.

2.7.2. *Water activity (Aw)*

The water activity was measured at 25 ºC using an Aqualab 4 TE (Decagon Devices, Pullman, WA, USA).

2.7.3. Hygroscopicity

The hygroscopicity was determined according to the methodology proposed by Cai & Corke (2000) with modifications. One gram of sample was placed in a desiccator containing a saturated sodium chloride solution (relative humidity 75.3%) at 25 ºC. After one week the samples were weighed and the hygroscopicity expressed in g of moisture absorbed by 100 g sample on a dry weight basis (g 100 g^{-1}).

2.7.4. *Color analysis*

The microcapsule color was analyzed using a previously calibrated Minolta Chroma Meter CR-400 colorimeter (Osaka, Japan), adjusted to operate with the D65 illuminant and 10º hue. The CIELab color scale was used to measure the parameters L*, a* and b*, where L* represents the luminosity from black (0) to white (100), a* represents the color variation from green (-) to red (+) and b* represents the color variation from blue (-) to yellow (+).

2.8. Evaluation of microcapsule stability and the in vitro release of bifidobacteria

The microparticles were added with probiotic initial count of 7.49 log CFU g^{-1} to three different buffer solutions: phosphate buffer pH 7.5, phosphate buffer pH 6.0 and acetate buffer pH 4.5. The mixtures were shaken at 150 rpm and 37 ºC in a model TE 421 (Tecnal, Piracicaba, SP, Brazil) controlled temperature shaker and samples removed after 60, 120 and 180 minutes to determine the release of *Bifidobacterium* BB-12 from the microcapsules. The integrity of the particles was monitored by optical microscopy before and after addition of the buffers at the same time intervals as the samples taken for the microbiological analyses.

2.9. Evaluation of the survival of free and encapsulated Bifidobacterium BB-12 exposed to simulated gastrointestinal conditions

The method proposed by Madureira et al. (2011) was used to simulate the gastrointestinal conditions, with modifications. The viability of the bacterium was evaluated sequentially in media simulating different sections of the gastrointestinal tract (esophagus/stomach, duodenum and ileum). For the step simulating passage through the esophagus/stomach, a solution containing 25 mg mL^{-1} of pepsin (Sigma, Germany) prepared in 0.1 M HCl was added together with 1 g of microcapsules or 1 g of freeze dried culture. The solution was added in equal aliquots, at a rate of 0.05 mL mL^{-1} during 90 minutes, the pH being adjusted to 2.0 with 1M HCl. For the step simulating passage through the duodenum, a solution containing 2 g L^{-1} pancreatin (Sigma) and 12 g L^{-1} bovine bile salts (Sigma) prepared in 0.1 M $NaHCO_3$ and adjusted to pH 5.0 was used. Finally for the step simulating passage through the ileum, the pH was increased to 6.5 using a 0.1 M $NaHCO_3$ solution. All the solutions were prepared on the day of analysis and sterilized by filtration through membranes with a pore size of 0.20 μm (Millipore, Billerica, MA, USA). The analysis was carried out in

a model TE 421 (Tecnal, Piracicaba, SP, Brazil) controlled temperature shaker maintained at 37 ºC with the objective of simulating body temperature. Mechanical shaking was carried out in parallel with different intensities (130 rpm for the esophagus/stomach, and 45 rpm for the duodenum and ileum) so as to simulate the peristaltic movements in each section of the digestive tract. Aliquots were removed after 0 min, 90 min (esophagus/stomach), 110 min (duodenum) and 200 min (ileum) to determine the survival of free and microencapsulated *Bifidobacterium* BB-12 by enumeration in MRS agar, as used to determine the survival of microencapsulated bifidobacteria in section 2.5.

2.10. Viability of Bifidobacterium BB-12 at different storage temperatures

The viability of the microencapsulated microorganisms was determined by enumeration in MRS agar as described in section 2.5 after storage for 0, 15, 30, 45, 60, 75, 90, 105 and 120 days at -18 °C, 7 °C and 25 °C.

2.11. Statistical analyses

The data were submitted to an analysis of variance (ANOVA) using the Statistica version 7.0 software (2004) (Statsoft Inc., Tulsa, OK, USA), followed by Tukey's means comparison test at a level of 5% significance when significant differences between the treatments were noted. All the tests were carried out in triplicate and the data expressed as the mean ± standard deviation.

3. Results and discussion

3.1. Encapsulation efficiency

With respect to encapsulation efficiency (%EE) the results presented in Table 1 show a mean value of 89.71%. The encapsulation efficiency found in the present study was equivalent to the external ionic gelation process, which gave %EE values in the range from 80-98% using the same wall material of sodium alginate (Martin et al., 2015). However in the present research, the efficiency was shown to be greater than that found in the studies of Zou et al. (2011) who microencapsulated *Bifidobacterium bifidum* F-35 and Song, Yu, Gao, Liu & Ma (2013) who encapsulated the Y235 yeast cells, and obtained encapsulation efficiencies of

43-50% and 70%, respectively, both using alginate microcapsules prepared by a technique similar to emulsification/internal ionic gelation. This indicates that the microorganisms did not suffer pronounced damage during the microencapsulation process, showing it to be a feasible and adequate technique to produce microcapsules containing probiotics.

Table 1
Characteristics of the microcapsules of *Bifidobacterium* BB-12 obtained by internal ionic gelation with alginate

	Microparticles with alginate*
Particles size (μm)	77.84 ± 0.54
Water activity - Aw	0.20 ± 0.01
Hygroscopicity (g $100g^{-1}$)	2.00 ± 0.5
Colour attributes	
L*	70.53 ± 5.21
a*	-0.36 ± 0.36
b*	18.90 ± 8.02
Encapsulation yield (EY%)	89.71 ± 9.50

* Results expressed as mean ± standard deviation.

3.2. Characterization of the microcapsules

The microcapsules had an elliptical shape with multi-cavities distributed throughout them and a wrinkled surface (Fig. 1a). The same was reported by Huang & Fu (2010) who produced freeze dried gelatin microcapsules and detected the presence of cavities uniformly distributed on the surface as well as wrinkles. These effects on the particle surfaces are due to the rapid sublimation of frozen water from the alginate matrix, resulting in the formation of cavities where there were ice crystals before (Smrdel, Bogataj, Zega, Planinsek, & Mrhar, 2008). The results also showed the formation of particle agglomerates in all the samples (Figure 1b), corroborating with the study of Salvim et al. (2015) who produced lipid microcapsules with soy protein hydrolysate. In this study it was observed that the microcapsules with smaller diameters adhered to the surfaces of larger particles. Similar shaped microcapsules were also obtained by Zou et al. (2011) and Castro-Cislaghi et al. (2012).

The particle size is a physical parameter of importance since it can influence the aroma, texture and appearance of the food, particle sizes above 100 μm being desirable, so as to avoid a negative impact on the sensory characteristics (Burgain et al., 2011). In the present study the microcapsules presented mean diameters of 77.84 μm (Table 1) which is below this

value. The present study obtained smaller diameters than those obtained in the study of Cai et al. (2014), who microencapsulated *Lactobacillus acidophilus* in alginate by emulsification and obtained particles with mean sizes in the range from 323 to 343 µm. On the other hand, Yúfera, Fernández-Díaz & Pascual (2005) prepared microcapsules with micro-diets for fish larvae by internal gelation and obtained a mean particle size of 75 µm, corroborating with the present study.

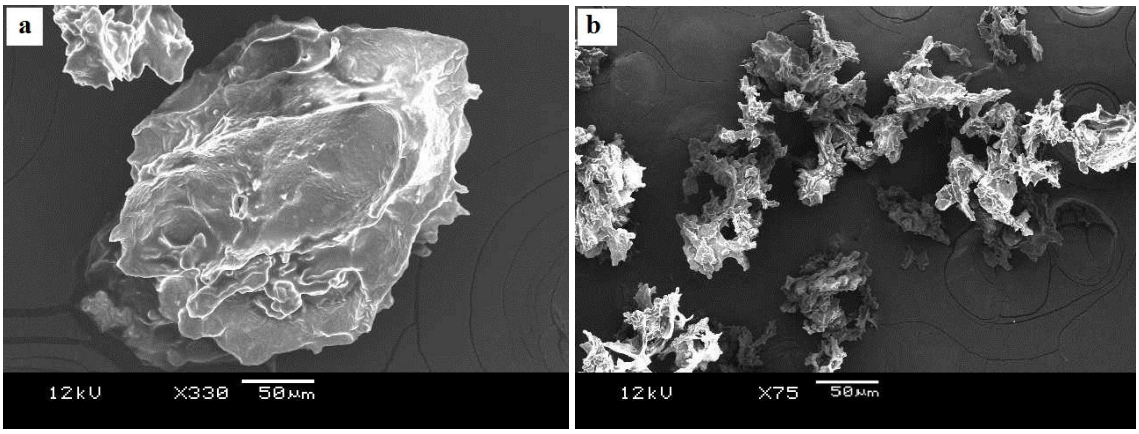

Fig. 1. Micrographs of microcapsules of *Bifidobacterium* BB-12. (a) 330×; (b) 75×.

The moisture content of freeze dried probiotic microcapsules is a critical factor that influences the stability of the bacteria during storage, but generally the microorganisms survive better at low water activity values (Chan et al., 2011; Golowczyc et al., 2010; Meng et al., 2008). The microcapsules showed a water activity of 0.2 (Table 1), which, according to Tonon et al. (2009) is highly positive for the stability of the microcapsules, with little free water available for biochemical reactions, consequently prolonging the shelf life. Similar results were obtained by Fritzen-Freire et al. (2012) who evaluated the viability and physicochemical properties of microcapsules containing *Bifidobacterium* BB-12, obtained by spray drying with reconstituted milk and inulin. The results of this study allowed for the conclusion that all the microcapsules presented values for water activity (Aw) below 0.3, conferring greater stability. Thus the values for Aw obtained in the present study for the microcapsules of *Bifidobacterium* BB-12, produced with alginate were appropriate for long term storage.

Hygroscopicity is the capacity of the microcapsules to absorb moisture from an environment with a high relative humidity, and the viability of the microorganisms is affected by the relative humidity conditions during storage (Bhandari et al., 2013). Table 1 shows that

the microcapsules showed a mean hygroscopicity of 2.00 g 100 g^{-1}, due to the formation of a pellicle with the oil residue on the particle surface, reducing the exposed area (cavities and pores), which, consequently, resulted in less moisture absorption. This also occurred in the study of Rajam & Anandharamakrishnan (2015) who microencapsulated *Lactobacillus plantarum* with whey proteins and sodium alginate by spray freeze drying method was performed in two steps namely spray freezing followed by freeze drying. They concluded that the formation of the pellicle on the surface of the microcapsules by the encapsulating agents, alginate, whey protein and fructooligosaccharide, resulted in the formation of particles with low hygroscopicity.

Table 1 also shows the color parameters of the microcapsules containing *Bifidobacterium* BB-12. The parameter L* (luminosity) was quite high indicating that the particles were light colored. With respect to the parameters a* and b*, the microcapsules showed a negative value for a* and positive value for b*, indicating a tendency for the colors green and yellow, respectively. Such behavior was expected since the use of rapeseed oil, which shows a hue between yellow and green, interfered with their color. In the same way, sodium alginate, which shows a hue between white and yellow, also contributed to the intensity of the yellow color. These results corroborated with the study of Aryana & McGrew (2007), who added *Lactobacillus casei* and inulin to yogurt, and observed that the color of the ingredients added, altered the color of the final product, conferring a whitish aspect due to the use of the prebiotic.

3.3. Evaluation of microcapsule stability and the in vitro release of Bifidobacterium BB-12

Table 2 shows the release of the microorganisms *Bifidobacterium* BB-12 from the particles after the trials in different buffer systems. The counts were lower at pH 4.5, varying from 0.92 to 1.01 log CFU g^{-1} at the times tested. This signified there was practically no release or dissolution of the particles in this medium, and this count probably occurred due to free cells on the particle surface. In the pH 6.0 buffer the counts were intermediate, remaining between 5.40 and 5.85 log CFU g^{-1}, demonstrating partial dissolution and release of the probiotic cultures. Finally the highest counts were obtained in the pH 7.5 buffer, reaching 6.71 log CFU g^{-1} after 180 minutes.

Table 2
Populations of *Bifidobacterium* BB-12 in the microcapsules obtained by internal ionic gelation treated with solutions pH 4.5, 6.0 and 7.5

Time (minutes)	pH		
	4.5	6.0	7.5
60	$0.92^{aA} \pm 0.80$	$5.40^{aB} \pm 0.25$	$5.88^{aB} \pm 0.04$
120	$0.98^{aA} \pm 0.85$	$5.72^{aB} \pm 0.11$	$6.33^{bB} \pm 0.07$
180	$1.01^{aA} \pm 0.88$	$5.85^{aB} \pm 0.12$	$6.71^{cB} \pm 0.08$

[a-c]Within a column, different superscript lowercase letters denote significant differences ($p < 0.05$) among the different incubation times in the same pH.
[A-C] Within a line, different superscript uppercase letters denote significant differences ($p < 0.05$) among the different pH values in the same incubation time.

In the buffer systems at pH 4.5 and 6.0, no significant differences ($p > 0.05$) were observed between the times, since rupture of the microcapsules was slow, producing low counts. On the other hand in the pH 7.5 buffer system the results were significantly different ($p < 0.05$) due to the gradual and rapid release up to 180 minutes of incubation. These results are in agreement with those of Yotsuyanagi et al. (1987), who studied the formation of the gel induced by calcium in sodium alginate and the stability of these particles in acid solution. From the results they concluded that these microcapsules were resistant, making it possible to protect an active compound. In another study carried out by Sugawara, Imai & Otagiri (1994), they encapsulated prednisolone (drug) in sodium alginate particles, and observed greater release above pH 6.0. The release profile shows that these particles possess the potential for application in foods with pH values below 4.5, and at the same time allowing for their controlled release in the intestine, since the hydrated alginate is converted into an insoluble layer of porous alginic acid, which, at higher pH values, dissolves, releasing the core material (George & Abraham, 2006).

3.4. Evaluation of the survival of free and microencapsulated Bifidobacterium BB-12 exposed to simulated gastrointestinal conditions

Fig. 2 shows the results for the survival of *Bifidobacterium* BB-12 exposed to simulated gastrointestinal conditions, both for the free cells and those microencapsulated with sodium alginate by emulsification/internal ionic gelation. The initial counts before the addition of the simulated gastrointestinal system were 11.91 log CFU g^{-1} for the microencapsulated microorganisms and 14.53 log CFU g^{-1} for the free cells. The initial viable counts were in agreement with the minimum values recommended for the addition to a probiotic product, as suggested by Aureli et al. (2011) and Salminen, Kenifel & Ouwehand

(2011), who stipulated that the ingestion of probiotic cells should be around 8 – 9 log CFU g^{-1} in order to obtain beneficial effects on the health.

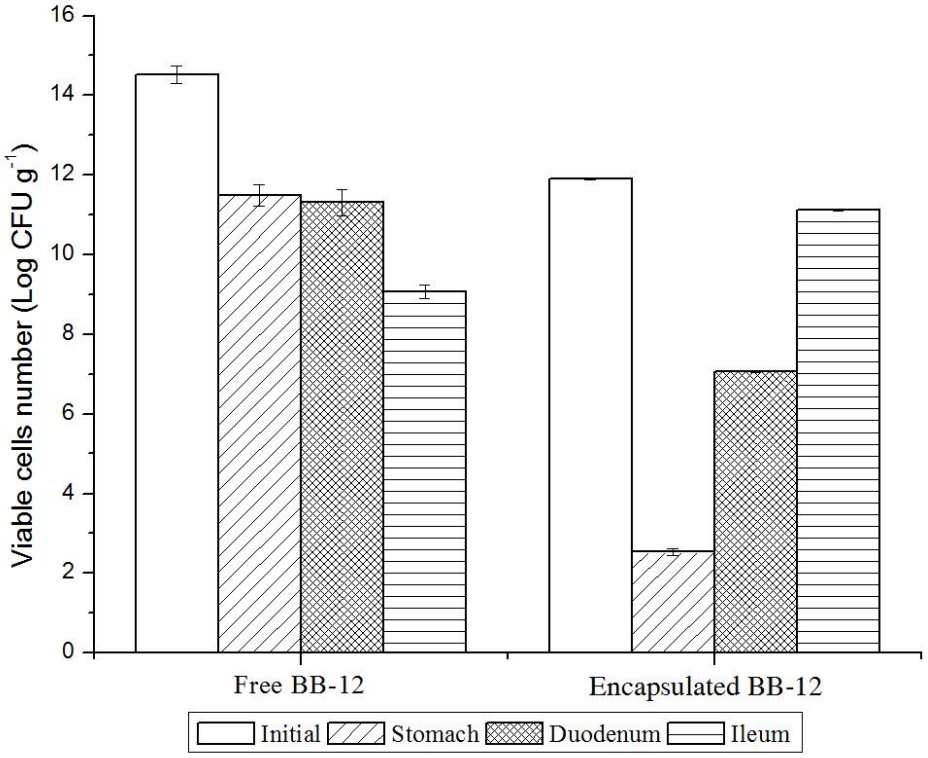

Fig. 2. Survival of *Bifidobacterium* BB-12 free and encapsulated after each step of the simulated gastrointestinal conditions.

After 90 minutes of incubation, the hydrogenionic potential was gradually adjusted with a pepsin solution until it reached a value of 2.0 (esophagus/stomach simulation). This caused a decrease of 3.04 log CFU g^{-1} (p<0.05) in the free cells as compared to the initial count due to the low pH value at this step in the process, resulting in low viability of the probiotic bacteria. These results corroborated those of Madureira et al. (2005), who evaluated the survival of free probiotic bacteria in cheese, and observed a decrease of approximately 2 logarithmic cycles in the viability. The same occurred in the study of Verruck et al. (2015), who added *Bifidobacterium* BB-12 to fresh Minas cheese made from buffalo milk, and showed a significant decrease in viability after the simulation of stomach conditions.

After exposure of the microencapsulated cells to acid conditions (esophagus/stomach simulation), a count of 2.54 log CFU g^{-1} was obtained, a difference of 9.37 log CFU g^{-1} when compared to the initial count of 11.91 log CFU g^{-1}. However this difference did not refer to a loss of viability as in the case of the free cells, since in this case it was noted that the microcapsules did not rupture, and consequently the probiotic bacteria were not released at a

pH value of 2.0. These results corroborate with those of Doleyres & Lacroix, 2004 and Ding & Shah, 2007, who microencapsulated different probiotics (*Lactobacillus rhamnosus*, *Bifidobacterium longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. paracasei* and *B. lactis)* and observed that the sodium alginate microcapsules protected the microorganisms under acid conditions.

Fig. 2 shows the results for the counts of viable bifidobacterial cells in the microcapsules after exposure to conditions simulating those of the duodenum (that is, bile salts, pancreatin and pH 5.0). Under these conditions counts of 7.07 log CFU g^{-1} ($p < 0.05$) were obtained, showing that conditions simulating those of the duodenum allowed the majority of the microcapsules to rupture, with a consequent increase in the cell count as compared to the acid conditions. On the other hand the free cells showed no significant difference, although a slight decrease of 0.17 log CFU g^{-1} ($p > 0.05$) was registered in the count. This occurred due to the damage caused to the cells by the acid conditions to which they were previously exposed, corroborating with the study of Chávarri et al. (2010), who microencapsulated probiotics with chitosan and alginate by extrusion, and concluded that survival was greater in the bile salts, with a survival rate of about 96% of the microencapsulated bacteria as compared to the free cells. In another study, Rajam et al. (2012) microencapsulated *Lactobacillus plantarum* with protein isolate and alginate by spray drying and concluded that the viability increased significantly after the addition of the cultures to conditions at pH 5.0 with bile salts.

After submitting the microcapsules to conditions simulating those of the ileum (pH 6.5), they showed a decrease of 0.78 log CFU g^{-1} as compared to the initial count before simulation ($p < 0.05$). Rupture of all the microcapsules was observed in this section of the simulated gastrointestinal tract, releasing the *Bifidobacterium* cells in their desired place. These results corroborate those of Etchepare et al. (2016), who microencapsulated *Lactobacillus acidophilus* in sodium alginate by extrusion, and concluded that higher probiotic counts were obtained at pH values above 6.0, with counts above 6 log CFU g^{-1}.

The results obtained for the free cell count showed a reduction of 5.46 log CFU g^{-1} ($p < 0.05$) as compared to the initial count before simulation. This occurred due to the variation in pH during simulation of the gastrointestinal tract, which caused a significant reduction in viability. Iyer & Kailasapathy (2005), who co-encapsulated probiotic *Lactobacillus acidophilus* with modified starches and simulated gastrointestinal conditions, presented results showing that the encapsulated probiotic bacteria survived in greater numbers than the free probiotic cells. Thus the results obtained indicated that the microencapsulation process

conferred protection on the probiotic bacteria during the gastrointestinal simulation, maintaining the viability of the probiotics.

3.5. Stability of the microencapsulated microorganisms during storage at different temperatures

Table 3 shows the effects of room temperature (25 ºC), refrigeration (7 ºC) and frozen (-18 ºC) storage during 120 days on the viability of *Bifidobacterium* BB-12 in the freeze dried microcapsules.

Table 3
Effect of room temperature (25 °C), freezing (-18 °C) and refrigeration (7 °C), on the viability of microencapsulated *Bifidobacterium* BB-12 stored for 120 days.

Time (days)	Temperature*		
	25 °C	-18 °C	7 °C
0	$8.36^{aA} \pm 0.10$	$8.90^{bA} \pm 0.05$	$8.99^{bA} \pm 0.04$
15	$7.91^{aB} \pm 0.27$	$8.83^{bA} \pm 0.08$	$8.26^{aB} \pm 0.22$
30	$7.11^{aC} \pm 0.01$	$8.91^{bA} \pm 0.03$	$7.02^{aC} \pm 0.08$
45	$6.08^{aD} \pm 0.03$	$8.06^{bB} \pm 0.08$	$6.17^{aD} \pm 0.32$
60	$7.88^{aB} \pm 0.05$	$9.14^{bC} \pm 0.06$	$8.31^{cB} \pm 0.25$
75	$3.92^{aE} \pm 0.02$	$7.60^{bD} \pm 0.05$	$7.54^{bC} \pm 0.32$
90	$3.83^{aE} \pm 0.08$	$7.48^{bDE} \pm 0.03$	$7.63^{bC} \pm 0.10$
105	$2.92^{aF} \pm 0.04$	$7.31^{bEF} \pm 0.08$	$7.32^{bC} \pm 0.23$
120	$2.10^{aG} \pm 0.01$	$7.19^{bF} \pm 0.06$	$7.32^{bC} \pm 0.12$

* Results expressed as mean ± standard deviation.
a-c Within a line, different superscript lowercase letters denote significant differences ($p < 0.05$).
A-G Within a column, different superscript uppercase letters denote significant differences ($p < 0.05$).

During room temperature storage, the number of viable cells remained above 6 log CFU g^{-1} for up to 60 days. After this period there was a significant reduction ($p < 0.05$) of 5.78 log CFU g^{-1} in the viability of these microorganisms during the last 60 days of storage. This could be explained by the fact that there was greater metabolic activity of the microorganisms, producing metabolic acids and bacteriocins and even showing a loss of substrates during storage. Similar results were found by Okuro et al. (2013) who microencapsulated *Lactobacillus acidophilus* in inulin by spray chilling, and showed that the viability remained constant for 60 days at a temperature of 22 ºC. The same occurred in the research of Teanpaisan et al. (2012), who evaluated the drying of skimmed milk containing

Lactobacillus paracasei by spray drying, and observed a rapid decrease in viability at a temperature of 25 ºC, reaching a value of 0% after 6 months of storage.

On the other hand the results for the viability of the probiotics stored at 7 ºC remained above 7 log CFU g^{-1} after 120 days of storage, showing a reduction of only 1.67 log CFU g^{-1}, and hence the cell viability was relatively constant. These results are similar to those of Albertini et al. (2010), who microencapsulated *Lactobacillus acidophilus* LA14 and *Bifidobacterium lactis* BI07 by extrusion and reached counts of 10^8 CFU g^{-1} after 6 months of storage at 5 ºC, with a reduction of only 1.6 log CFU g^{-1}. In contrast, Prisco et al. (2015), who encapsulated *Lactobacillus reuteri* with alginate and chitosan by vibration technology in an encapsulator, obtained a reduction of close to 1 log CFU g^{-1} in the viability after 28 days of storage at 4 ºC, whereas in the present study the loss was practically the same after a longer shelf life. Frozen storage maintained the viability of the probiotics at 7.19 log CFU g^{-1} after 120 days of storage at -18 ºC. Similar results were obtained by Homayouni et al. (2008) who microencapsulated *Lactobacillus casei (Lc-01)* and *Bifidobacterium lactis (BB-12*) by emulsification with resistant starch, and added the product to ice cream, obtaining a reduction close to 1 log CFU g^{-1} after 180 days storage at -20 ºC. Similar results were obtained by researchers using other encapsulation techniques, such as the study of Pinto et al. (2015), who maintained the viability of bifidobacteria microencapsulated in skimmed milk plus inulin by the technique of spray drying, at a count above 6 log CFU g^{-1} for 90 days of frozen storage. In this study there was no significant difference between refrigerated and frozen storage, suggesting that the stability of the cells increased with decrease in temperature, since crystal rearrangement and exposure of the active ingredient are impeded at low temperatures, promoting a longer shelf life of the microcapsules. The same occurred for Corcoran et al. (2004), who evaluated the survival of *Lactobacillus rhamnosus* with the prebiotic (inulin) and observed that the probiotic microorganisms survived longer at low temperature.

The results suggest that the application of freeze drying soon after microencapsulation constitutes a feasible alternative, with the objective of eliminating residual water and maintaining the viability of probiotic cultures both at room temperature for up to 60 days and at 7 ºC and -20 ºC for up to 120 days.

4. Conclusions

The technique of emulsification/internal ionic gelation together with freeze drying was shown to be feasible and efficient to increase the viability of probiotic cultures. The results demonstrated that *Bifidobacterium* BB-12 resists the process of microencapsulation in alginate microcapsules and that the encapsulation process was efficient in protecting the probiotics in their passage through simulated gastrointestinal fluids and under acid conditions. The particles were also stable during storage at 7 ºC and -18 ºC for 120 days and at room temperature (25 ºC) for up to 60 days of storage.

In addition, the morphology, size, hygroscopicity, color and encapsulation efficiency of the microcapsules were all highly positive, offering advantages compared to other encapsulation techniques, which could make their incorporation into food products easier and feasible, with little interference with respect to the sensory aspects of the foods.

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7 DISCUSSÃO

Durante o processo de microencapsulação é necessário o conhecimento dos parâmetros que influenciam as características das micropartículas. A natureza e a concentração destes fatores influenciam a distribuição e o tamanho da partícula e consequentemente na viabilidade do microrganismo (AHMED et al., 2013; MARTIN et al., 2013). Antes da produção das microcápsulas foram realizados testes preliminares a fim de otimizar o processo de encapsulação sobre três variáveis importantes: concentração de alginato de sódio, velocidade de rotação e concentração de emulsificante com relação ao diâmetro das micropartículas e eficiência de encapsulação.

A otimização do processo sugeriu um deslocamento das condições operacionais para a região de elevada concentração de emulsificante (1.5%) e elevada velocidade de rotação (900 rpm) com uma concentração menor de alginato de sódio (1,5%) a fim de obter diâmetros médios de 55 µm. Os resultados indicam que a eficiência de encapsulação se apresentou superior a 90% com viabilidade da cultura probiótica (13,98 UFC g^{-1}) na região considerada ótima neste estudo. Desse modo, definiu-se estas concentrações para produção e a avaliação das micropartículas.

7.1 Caracterização das microcápsulas

7.1.1 Análises físico-químicas

As análises físico-químicas apresentaram uma elevada umidade das micropartículas em torno de 96.17% ± 1.82. Diversas pesquisam justificam que esses valores elevados são característicos de micropartículas produzidas com polissacarídeos formadores de géis, devido a sua elevada capacidade de retenção água (AGUILAR et al., 2015). Os resultados apresentaram cerca de 2.24% de perfil lipídico, esta quantidade é originária do óleo que apesar de quantidades reduzidas ainda está presente na superfície da cápsula decorrente da técnica de encapsulação. As micropartículas apresentaram em média de 0.56% de matéria

mineral, devido a presença de cálcio insolúvel no início de processo e uma quantidade de carboidratos de 1.08% originário do alginato de sódio que foi o material de parede utilizado.

O teor de água e a higroscopicidade em micropartículas de probióticos liofilizadas é um fator crítico que influencia a estabilidade das bactérias durante o armazenamento. As microcápsulas mostraram uma água atividade de 0.2 que de acordo com a Tonon et al. (2009) é muito positivo para a estabilidade das micropartículas, pois existe menos água livre disponível para reações bioquímicas consequentemente prolongando a vida útil. As partículas apresentaram uma menor higroscopicidade na média de 2.00 g 100 g^{-1}, isso ocorreu pela tendência de formação de uma película com o resíduo do óleo sobre a superfície exterior da partícula reduzindo a área exposta (cavidades e poros) que consequentemente resultou em menos absorção de umidade (RAJAM & ANANDHARAMAKRISHNAN, 2015).

7.1.2 Morfologia e tamanho das micropartículas

Através da microscopia ótica foi possível observar a morfologia externa e a estrutura interna das microcápsulas produzidas, o tamanho e a distribuição de recheio (microrganismos) na matriz. Em geral, as microcápsulas úmidas apresentaram forma esférica em sua maioria e distribuição do recheio por toda a matriz. Não foi observada agregação das micropartículas, pela quantidade de óleo que possivelmente estava presente na superfície que evitou a aglomeração entre elas. Porém, a estrutura das micropartículas liofilizadas foi observada em microscópio eletrônico de varredura (MEV), apresentando uma forma elíptica com multicavidades distribuídas e uma superfície com aspecto rugoso. Estes efeitos na superfície das partículas, provavelmente são causados pela sublimação rápida da água congelada a partir de matriz de alginato, resultando na formação de cavidades em áreas onde estavam os cristais de gelo (SOUSA et al., 2015).

Em relação ao diâmetro, as micropartículas úmidas apresentaram diâmetros inferiores a 60 μm, enquanto as liofilizadas apresentaram diâmetros maiores que 70 μm, em função da hidratação e intumescimento. Diâmetros menores que 100 μm são os desejáveis para a maioria das aplicações, pois oferece uma melhor proteção no suco gástrico e apresentam bom aspecto sensorial quando incorporados em alimentos (ARUP et al., 2011).

7.1.3 Análise de cor

Os parâmetros de cor tanto para as micropartículas úmidas como para as micropartículas liofilizadas foram semelhantes. O parâmetro L* (luminosidade) foi um pouco elevado indicando que as micropartículas apresentaram cor clara. Com relação aos parâmetros a* e b*, as microcápsulas apresentaram valor negativo para a* e positivo para b*, indicando uma tendência às colorações verde e amarela, respectivamente. Tal comportamento era esperado uma vez que, a utilização do óleo de canola que possui uma tonalidade entre o amarelo e verde interferiu na cor das mesmas. Do mesmo modo, o alginato de sódio que possui uma tonalidade entre o branco e amarelo também contribui para a intensidade da cor amarelo. Atualmente tem sido relatada uma grande perda de microrganismos probióticos na produção de queijos, e neste sentido, a incorporação destas micropartículas em um produto que possua a mesma tonalidade de cor, surge como uma alternativa viável (ROSAS-FLORES et al., 2013).

7.2 Avaliação da sobrevivência de *Bifidobacterium* BB-12 livre e microencapsulado exposto a condições gastrointestinais simuladas

Na avaliação da sobrevivência dos probióticos às condições gastrointestinais simuladas pode-se perceber que as bifidobactérias livres nas condições simuladas do íleo (pH 6.5), ocorreu uma redução maior que 5 log UFC g^{-1} comparada com a contagem inicial antes da simulação. Isso mostra que a exposição das células livres em condições ácidas e enzimas gástricas resultaram em um decréscimo significativo sobre a viabilidade das bactérias probióticas. Isto está de acordo com outros estudos que mostram que *Bifidobacterium* BB-12 é uma cepa sensível ao ácido (RANADHEERA et al., 2015).

Quanto a viabilidade das micropartículas úmidas, a exposição das células microencapsuladas em condições ácidas (simulação do esôfago/estômago) obteve-se um resultado de 4.32 log UFC g^{-1} comparado com a contagem inicial antes da simulação que foi de 13.42 log UFC g^{-1}, pois não houve rompimento das micropartículas. Após as condições simuladas do íleo (pH 6.5), as partículas apresentaram uma diminuição de 0.49 log UFC g^{-1} comparada com a contagem inicial antes da simulação ($p < 0,05$) isto se deve ao fato que, o

processo de microencapsulação conferiu proteção as bactérias probióticas na simulação gastrointestinal. O mesmo foi encontrado no estudo de Iyer & Kailasapathy (2005) que coencapsularam probiótico *Lactobacillus acidophilus* com amidos modificados que simularam as condições gastrointestinais apresentaram resultados onde as bactérias probióticas encapsuladas sobreviveram em números mais elevados do que as células probióticas livres.

Já nas microcápsulas na forma liofilizada, observou-se que após a exposição a condições gástricas simuladas em pH ácido, a população de *Bifidobacterium* BB-12 apresentou contagens reduzidas, demonstrando assim que, provavelmente não houve liberação da cápsula. Após as condições simuladas do íleo (pH 6.5), as micropartículas apresentaram uma diminuição de 0.78 log UFC g^{-1} comparada com a contagem inicial antes da simulação ($p < 0,05$). Foi observado que ocorreu o rompimento de todas as micropartículas nesta seção do trato gastrointestinal simulado, liberando as células probióticas no seu local desejado. Corroborando com o estudo de Xu et al. (2016) que microencapsularam *Lactobacillus casei* ATCC 393 por extrusão e *freeze-drying* obtendo o número de células viáveis estabilizado (0,03 log UFC g^{-1}) durante o período de simulação gastrointestinal.

A microencapsulação de *Bifidobacterium* BB-12 tanto na forma úmida como liofilizada ofereceu um meio eficaz de entrega de células bacterianas viáveis a nível intestinal, em condições apropriadas e ajuda a manter a sua sobrevivência durante a passagem simulada pelo trânsito gastrointestinal. A utilização de alginato de sódio como material de parede, o qual é de natureza não toxica e boa capacidade de revestimento, retardou a liberação dos probióticos, para que a mesma não ocorresse no estômago e sim no intestino (AMINE et al., 2014).

Entretanto, na forma liofilizada houve uma redução 1.80 log UFC g^{-1} comparado as micropartículas úmidas depois da simulação. Santivarangkna et al. (2008) estudaram os efeitos da congelação, desidratação e armazenagem na funcionalidade das membranas celulares. A ruptura das membranas celulares devido ao processo de desidratação aumentou a sensibilidade das células aos íons. Além disso, a remoção da umidade em células durante o processo de secagem combinado oxidação lipídica durante o armazenamento pode influenciar tanto a fluidez e as propriedades das membranas celulares (KIM et al., 2008). Desse modo, sugere-se a necessidade da adição de um agente crioprotetor e/ou demais adjuvantes no material de revestimento.

7.3 Avaliação da estabilidade das micropartículas e da liberação das bifidobactérias *in vitro*

As micropartículas úmidas e liofilizadas foram avaliadas no tampão pH 4,5 que se verificou contagens mais baixas em torno de 0.92 a 1.01 log UFC g^{-1}, respectivamente nos tempos testados. Isso significa que praticamente não houve liberação ou dissolução das partículas nesse meio, e essa contagem provavelmente ocorreu devido às células que estão na superfície das partículas e que podem se desprender a qualquer momento. No tampão pH 6,0, constatou-se que os valores de contagens foram intermediários permanecendo entre 5.40 e 5.85 log UFC g^{-1}, respectivamente. Provavelmente nesse meio a dissolução das partículas não foi completa. Finalmente, com o tampão pH 7,5, obteve-se as contagens mais altas, chegando a 6.71 log UFC g^{-1}, respectivamente após 180 minutos.

Estes resultados equivalem ao estudo de Déat-Lainé et al. (2012) que microencapsularam insulina com alginato e proteína por extrusão concluíram que em pH ácido ocorre uma liberação gradual do bioativo e no pH básico ocorre uma liberação brusca pela degradação dos polímeros. Yotsuyanagi et al. (1987) que pesquisaram a formação da gelificação induzida pelo cálcio em alginato de sódio e a estabilidade dessas partículas em solução ácida, concluíram que estas micropartículas foram resistentes possibilitando uma proteção em um composto ativo. Esse perfil de liberação mostra que essas partículas possuem potencial para aplicação em alimentos com pH abaixo de 4,5 e também para uma possível liberação controlada no intestino.

7.4 Viabilidade de *Bifidobacterium* BB-12 durante o armazenamento em diferentes temperaturas

Na avaliação da viabilidade dos probióticos microencapsulados durante o armazenamento, nas micropartículas úmidas, ocorreu uma redução logarítmica de 6.74 log UFC g^{-1} na temperatura de 25 °C após os 120 dias de armazenamento com uma viabilidade probiótica até 75 dias. Em temperatura ambiente ainda existe atividade metabólica, resultando assim na morte de células e perda de viabilidade (SOTO et al., 2011). Na refrigeração (7 °C) houve uma maior redução que foi de 10.52 log UFC g^{-1} comparado as outras temperaturas de

armazenamento. Esta perda na viabilidade durante o armazenamento pode estar relacionada com uma série de fatores, tais como a oxidação dos ácidos graxos, formação de radicais livres na presença de oxigênio e danos no DNA celular (PEDROSO et al., 2012). No entanto, as células microencapsuladas que foram armazenadas na temperatura de -18 °C apresentaram uma contagem de 7.31 log UFC g^{-1} após os 120 dias de armazenamento. Estes resultados sugerem que a na temperatura de congelamento ocorreu maior estabilidade das células, pois em temperaturas baixas ocorre o impedimento de rearranjos de cristais e exposição do ingrediente ativo promovendo uma vida útil mais longa das micropartículas (MARTÍN et al., 2015).

Na estocagem em temperatura ambiente das micropartículas liofilizadas, o número de células viáveis permaneceu acima de 6 log UFC g^{-1} por apenas 60 dias de armazenamento. Após este período, houve uma redução significativa ($p < 0,05$) de 5.78 log nos últimos 60 dias de estocagem sobre a viabilidade destes microrganismos. Isso pode ser explicado, pois há uma maior atividade metabólica dos microrganismos, produzindo ácidos metabólicos, bacteriocinas ou até mesmo perda de substratos durante o armazenamento (OKURO et al., 2013).

Por outro lado, os resultados da viabilidade dos probióticos para a temperatura de 7 °C e -18 °C nas micropartículas liofilizadas foram acima de 7 log UFC g^{-1} após 120 dias de estocagem. Neste estudo, não houve diferença significativa entre as temperaturas de refrigeração e congelamento sugerindo que a estabilidade das células aumenta com a diminuição da temperatura, pois em temperaturas baixas ocorre o impedimento de rearranjos de cristais e exposição do ingrediente ativo promovendo uma vida útil mais longa das micropartículas (RIBEIRO et al., 2014). Na pesquisa de Martín et al. (2015) que microencapsularam *Lactobacillus fermentum* com alginato de sódio por emulsificação, concluíram que na temperatura de -20 °C ocorreu uma redução de apenas 0.8 log UFC g^{-1} depois de 45 dias, permanecendo-se estável no restante do período de armazenamento. A partir destes resultados pode-se sugerir que a aplicação de um processo de liofilização logo após a microencapsulação parece ser uma boa alternativa, com objetivo de eliminar a água residual, mantendo a viabilidade, tanto a temperatura ambiente até 60 dias como nas temperaturas de 7 °C e -20 °C até 120 dias.

Em comparação com outras técnicas de encapsulação, observou-se que este estudo apresentou uma vida de prateleira superior as demais pesquisas. No estudo de Pedroso et al. (2012) microencapsularam *Bifidobacterium lactis* por *spray-chilling* concluindo que as células microencapsuladas que foram armazenados a 37 °C tinha uma vida útil inferior a 30

dias, e a vida de prateleira foi entre 30 e 60 dias quando as células foram armazenadas a 7 °C ou -18 °C apresentando uma contagem abaixo de 10^6 UFC g^{-1}. Na pesquisa de Su et al. (2011) que microencapsularam *Bifidobacterium longum* BIOMA 5920 com 1,5% de alginato de sódio por extrusão, mostraram a estabilidade do livre e encapsulado *B. longum* BIOMA 5920 durante 3 semanas de armazenamento a 4 °C. O número de células livres diminuiu de 9.83 para 5.96 log UFC ml^{-1} e a sobrevivência do probiótico microencapsulado diminuiu de 9.07 para 6.65 log UFC ml^{-1}. Oliveira (2006) relatou que a viabilidade de *B. lactis* microencapsulado por meio de coacervação, seguido por secagem por pulverização com pectina e caseína começou a declinar dentro dos primeiros 30 dias de armazenamento sob refrigeração, resultando numa diminuição de 4.34 log após 120 dias.

8 CONCLUSÃO

Nas condições operacionais de 1,5% de alginato de sódio, 900 rpm de velocidade de rotação e 1,5% de emulsificante, as partículas apresentaram adequada distribuição de tamanho e uma eficiência de encapsulação superior a 90%. As micropartículas de *Bifidobacterium* BB-12 apresentaram características típicas, bem como bons diâmetros para a aplicação em alimentos. Tanto as micropartículas úmidas como as liofilizadas protegeram os microrganismos frente aos testes gastrointestinais simulados, ambas permitiram melhor liberação em pH intestinal, local onde os probióticos devem atuar.

Este estudo indicou uma eficiência média de 99,8% de encapsulação com a técnica de emulsificação/gelificação interna e pouca redução na viabilidade após a exposição em condições gastrointestinais. Porém, outras técnicas apresentam resultados de eficiência de encapsulação menores devido a temperatura, pH, porosidade da micropartícula entre outros fatores.

Em relação à viabilidade da cultura probiótica durante os períodos e temperaturas de armazenamento analisados, todos os tratamentos avaliados nas formas úmidas e liofilizadas foram viáveis demonstrando valores adequados para a ocorrência de efeitos probióticos, apresentando no mínimo 60 dias de estabilidade quando estocados na forma liofilizada em temperatura ambiente (25 °C) e 15 dias de viabilidade na forma úmida também na temperatura de refrigeração (7 °C). Portanto, este método inovador produz micropartículas estáveis para a aplicação de probióticos nos alimentos com baixo custo e um potencial aumento de escala industrial.

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