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UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

PRODUÇÃO E ENCAPSULAMENTO DE *Lactobacillus plantarum* E ESTUDOS DE ESTABILIDADE E APLICAÇÃO EM FORMULAÇÃO ALIMENTAR

Chaline Caren Coghetto

Porto Alegre

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Tese submetida ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos como um dos requisitos à obtenção do grau de Doutor em Ciência e Tecnologia de Alimentos.

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Porto Alegre (RS), Brasil

Novembro de 2015

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A banca examinadora, abaixo assinada, aprova a Tese "**PRODUÇÃO E ENCAPSULAMENTO DE *Lactobacillus plantarum* E ESTUDOS DE ESTABILIDADE E APLICAÇÃO EM FORMULAÇÃO ALIMENTAR**", elaborada por Chaline Caren Coghetto, como pré-requisito para obtenção do grau de Doutor em Ciência e Tecnologia de Alimentos.

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Dedico

Aos meus pais Sonimar e Eliane por terem me apoiado incondicionalmente em todas as minhas decisões, sem hesitar. Por todo amor e carinho, sempre me auxiliando e aconselhando, por me oferecer a mais sincera amizade que já conheci. Com todo meu amor, carinho e gratidão!

AGRADECIMENTOS

À minha família, em especial aos meus pais Sonimar e Eliane pelo imenso apoio em todos os momentos. Às minhas queridas avós, pelo amor, carinho e pelas orações.

Ao meu orientador Marco Antônio Záchia Ayub por permitir que fizesse parte de seu grupo de pesquisa, pelos conhecimentos compartilhados, todos os ensinamentos, conselhos e pela paciência.

À minha co-orientadora Graziela Brusch Brinques por todas as explicações minunciosas, por todo o tempo despendido me orientando, pela força e motivação que sempre me deste.

Ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos (ICTA) da UFRGS, pela oportunidade de realização desse trabalho.

À todos os colegas do Bioteclab, que além da ajuda em laboratório, fizeram com que o trabalho fosse bastante prazeroso através da amizade e carinho.

Às minhas queridas bolsistas de Iniciação Científica Julia Iankoski, Carolina Bettker Vasconcelos e Thais Lopes, por todo o auxílio e pelos momentos de descontração vivenciados.

Aos Professores do Instituto de Ciência e Tecnologia de Alimentos, em especial a Professora Simone Hickman Flores por toda a ajuda e ensinamentos.

A Professora Rosane Soares, Nataly Siqueira e Jéssica Pletsch pela parceria, colaboração e por possibilitar a utilização dos equipamentos do Instituto de Química.

À todos que de alguma forma contribuíram para a realização deste trabalho.

RESUMO

Os microrganismos probióticos são considerados suplementos alimentares vivos, apresentando benefícios ao hospedeiro e melhorando o balanço intestinal. A produção de *Lactobacillus* com alta densidade celular vem sendo estudada e possui grande interesse por parte da indústria, bem como o estudo de novos meios de cultivo alternativos. Outros interesses são a melhora da sobrevivência dos microrganismos durante a passagem pelo trato gastrintestinal por meio da microencapsulação e a elaboração de um produto com potencial probiótico que não necessite da cadeia do frio. Dentro deste contexto o presente trabalho objetivou a produção de microrganismo potencialmente probiótico em meio de cultivo vegetal e após microencapsulado, para obtenção de um pó alimentício para ser diretamente utilizado em alimentos. Na primeira etapa deste trabalho foi realizada uma avaliação de variáveis para fixar os parâmetros de processo e o meio de cultivo em biorreator submerso, para produção de biomassa de *Lactobacillus plantarum* BL011. O meio de cultivo e parâmetros de processo que apresentaram os melhores resultados para a produção de biomassa e ácido láctico foram: 40 g L^{-1} de açúcares totais (soro ácido de soja); 15 g L^{-1} de extrato de levedura; velocidade de agitação de 200 rpm; 25 °C e 4,5 vvm. Os resultados obtidos permitiram uma produção de biomassa de 17,87 g L^{-1} e 37,59 g L^{-1} de ácido láctico. Em uma segunda etapa deste trabalho o microrganismo foi microencapsulado pela técnica de electrospraying, utilizando como agentes encapsulantes alginato de sódio (ALG) e uma mistura de alginato de sódio e pectina cítrica (ALG-PEC). As células microbianas livres e microencapsuladas foram submetidas ao suco gástrico simulado (SGS) e suco intestinal simulado (SIS). O microrganismo controle (células livres) demonstrou uma diminuição de 6 e 4,2 log UFC mL^{-1} depois de 120 min de exposição, respectivamente. No entanto, as células microencapsuladas em ALG e em ALG-PEC apresentaram resistência considerável, diminuindo 2,9 log UFC mL^{-1} para SGS e 2,7 log UFC mL^{-1} para SIS. Testes de armazenamento sob temperatura de refrigeração por 21 dias apresentaram boa sobrevivência bacteriana de 9,3 log UFC mL^{-1} (ALG) e 8,6 log UFC mL^{-1} (ALG-PEC) para células microencapsuladas, enquanto que as células livres apresentaram uma sobrevivência de apenas 1,2 log UFC mL^{-1}. Na terceira etapa foram realizados experimentos para obtenção do pó alimentício com potencial probiótico, onde o microrganismo microencapsulado em ALG foi liofilizado e analisada a viabilidade no período de 6 meses de armazenamento a temperatura ambiente (25 °C), a

qual foi mantida acima de 7 log UFC g^{-1} de pó alimentício, a análise microbiológica (conforme legislação brasileira) realizada antes e após o período de armazenamento não demonstrou contaminações para os patógenos avaliados. Realizou-se uma análise sensorial adicionando o pó alimentício em suco natural de laranja, obtendo aceitação sensorial elevada, maior que 88 %. O suco com adição do pó alimentício foi exposto aos SGS e SIS e apresentou, após 120 min, redução de apenas 2,4 log UFC mL^{-1} para SGS e 1,3 log UFC mL^{-1} para SIS.

Palavras-chave: *L. plantarum* BL011; planejamento experimental; resíduo agro-industrial; microencapsulação; produto potencialmente probiótico.

ABSTRACT

Probiotic microorganisms are considered living dietary supplements showing benefic effects to hosts by improving the intestinal balance. The high cell density production of *Lactobacillus* has been the interest of many studies and presents great interest for industry, along with the development of new alternative culture media. Other concerns are the improvement of the survival of microorganisms during passage through the gastrointestinal tract by means of microencapsulation, and the preparation of a product with probiotic potential that would require no cold chain. In this context, this study aimed at producing potentially probiotic bacterium with alternative sources of cultivation substrates and its microencapsulation to obtain a food powder to be used directly in food. In the first step of this study a screnning of variables was carried out to set the process parameters and culture medium in the submerged bioreactor for the production of *L. plantarum* BL011. The optimized culture medium and processing parameters for biomass and lactic acid formation were: 40 g L^{-1} total sugar (liquid acid protein residue of soybean); 15 g L^{-1} yeast extract; stirring speed of 200 rpm; 25 °C, and 4.5 vvm. The results obtained allowed for a production of 17.87 g L^{-1} of biomass and 37.59 g L^{-1} of lactic acid. In a second step of this study *L. plantarum* BL011 was microencapsulated using the electrospraying technique, using as encapsulating agents sodium alginate (ALG) and a mixture of sodium alginate and citrus pectin (ALG-PEC). The free and microencapsulated cells were subjected to the simulated gastric juice (SGJ) and simulated intestinal juice (SIJ). The microorganism control (free cells) showed a decrease of 6 and 4.2 log CFU mL^{-1} after 120 min of exposure, respectively. However, the microencapsulated cells in ALG and in ALG-PEC showed significant resistance, decreasing by 2.9 log CFU mL^{-1} in SGJ, and 2.7 log CFU mL^{-1} in SIJ. Storage tests under refrigeration temperature for 21 days showed good bacterial survival of 9.3 log CFU mL^{-1} (ALG) and 8.6 log CFU mL^{-1} (ALG-PEC) for microencapsulated cells, whereas free cells showed a survival of only 1.2 log CFU mL^{-1}. In the third step of the work, it was obtained a food powder with probiotic potential, where the ALG-microencapsulated bacterium was lyophilized and viability was investigated within 6 months of storage at room temperature (25 °C), keeping 7 log CFU g^{-1} product of its initial value. Microbiological analyses (according to Brazilian legislation) performed before and after the storage period did not show any contaminations by pathogens. The formulated orange juice containing *L. plantarum* BL011 obtained high sensory

acceptance (> 88 %) in the sensory analysis. The juice with the addition of food powder was exposed to SGJ and SIJ and presented, after 120 min, reduction of 2.4 log CFU mL^{-1} for SGJ and 1.3 log CFU mL^{-1} for SIJ.

Keywords: *L. plantarum* BL011; Placket Burman; agro-industrial residue; microencapsulation; potencially probiotic product.

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NOMENCLATURA

Lista de símbolos

°C: grau Celcius

pH: potencial hidrogeniônico

mm: milímetro

Ca^{2+}: cálcio

Zn^{2+}: zinco

Cu^{2+}: cobre

g: grama

L: litro

h: hora

mL: mililitro

dm: decímetro

CaCl: cloreto de cálcio

min: minuto

g: força *g*

$MgSO_4 \cdot 7H_2O$: sulfato de magnésio

$MnSO_4 \cdot H_2O$: sulfato de manganês

rpm: rotações por minuto

M: molar

NaOH: hidróxido de sódio

H_3PO_4: ácido fosfórico

O_2: oxigênio

k_La: coeficiente de transferência de oxigênio

vvm: volume de ar por volume de meio por minuto

U: unidade

mM: milimolar

kV: kilovolts

cm: centímetro

keV: quiloelétrons-volt

COO^-: grupamento carboxílico

mg: miligrama

Hg: mercúrio

HCL: ácido clorídrico

α: alpha

β: beta

µm: micrômetro

µL: microlitro

Lista de abreviaturas e siglas

ADP: adenosina difosfato

ALG: alginato de sódio

ANOVA: Análise de variância

ANVISA: Agência Nacional de Vigilância Sanitária

AOAC: Associação Oficial de Química Analítica

ATP: adenosina trifosfato

ATR: Attenuated Total Reflectance

CFU: Colony Forming Unit

CMC: carboximetilcelulose

DNA: Ácido desoxirribonucleico

DOC: Dissolved oxygen concentration

EC: European Commission

EFSA: European Food Safety Authority

FAO: Food Agriculture Organization

FDA: diacetato de fluoresceína

FOS: fructooligossacharides

FTIR: Infrared Spectroscopy

G: ácido-α-Lgulurônico

GG: díades de guluronato

GRAS: Generally recognized as safe

HM: alto grau de metoxilação

HPLC: High-performance liquid chromatography

IMO: isomaltooligossacharides

LAB: bactéria ácido láctica

LAPRS: liquid acid protein residue of soybean

LM: baixo grau de metoxilação

LPMP: *L. plantarum* microencapsulated powder

M: ácido-β-D-manurônico

MM: díades de manuronato

MG/GM: hetero-díades

NAD/NADH: Dinucleótido de nicotinamida e adenina

NMP: número mais provável

PB: Plackett-Burman

PEC: pectina cítrica

QPS: Presunção de Segurança Qualificada

SEM: Scanning Electron Microscopy

SGJ: Simulated Gastric Juice

SIJ: Simulated Intestinal Juice

SLM: solid lipid microparticles

UFC: Unidade Formadora de Colônia

WHO: World Health Organization

WPI: whey protein isolate

INTRODUÇÃO

A obtenção de novos produtos alimentícios está cada vez mais desafiadora, tendo em vista que precisa agradar uma demanda dos consumidores que além de atrativos, também exigem produtos saudáveis (komatsu et al., 2008). Desta forma, observa-se o desenvolvimento de uma série de alimentos funcionais adicionados de componentes fisiologicamente ativos, como por exemplo, os probióticos (Annunziata; Vecchio, 2013).

Diversos compostos e alimentos têm sido enquadrados com o apelo de alimento funcional e dentre estes estão os probióticos. No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA), através da Resolução n.º 2 de 7 de janeiro de 2002 estabelece o conceito de probióticos. Segundo esta Resolução "probióticos são microrganismos vivos capazes de melhorar o equilíbrio microbiano intestinal produzindo efeitos benéficos à saúde do indivíduo" (Brasil, 2002).

Membros dos gêneros *Lactobacillus* e *Bifidobacterium* são os principais probióticos reconhecidos. *L. plantarum* é uma versátil bactéria ácido láctica (*lactic acid bacteria* - LAB) que é encontrada em uma grande variedade de ambientes. Este microrganismo tem uma longa história de uso seguro em produtos alimentícios sendo reconhecido e largamente empregado como probiótico (Bernardeau et al., 2008).

Tradicionalmente, probióticos são adicionados em produtos lácteos, tais como, leites fermentados, iogurtes (Iyer; Kailasapathy, 2005; Sultana et al., 2000; Amine et al., 2014), queijo (Fortin et al., 2011; Amine et al., 2014), e sorvete (Godward; Kailasapathy, 2003; Amine et al., 2014). No entanto, a intolerância à lactose, o teor de colesterol e alergias às proteínas do leite são grandes inconvenientes relacionados com os produtos lácteos fermentados, bem como o crescente número de consumidores vegetarianos em muitas partes do mundo (Prado et al., 2008; Nualkaekul et al., 2011;

Céspedes et al., 2013), demonstrando a necessidade da criação de produtos que contemplam este público alvo. Os alimentos probióticos vegetarianos precisam ser totalmente livres de ingredientes de origem animal para serem classificados como tais, consequentemente a bactéria probiótica deve ser cultivada em meio de crescimento isento de derivados animais para evitar a transferência destes constituintes para o produto final (Heenan et al., 2002).

Além disso, a maioria destes produtos oferecidos à população apresenta uma desvantagem que é a necessidade da manutenção da cadeia do frio até o consumidor final, dificultando assim a maior inserção de alimentos suplementados com probióticos. Desta forma, há uma demanda crescente por parte dos consumidores por produtos probióticos diversificados e que não requeiram refrigeração. Os custos inerentes à manutenção da cadeia do frio podem ser largamente reduzidos, ou até eliminados, através do desenvolvimento de produtos em pó secos que podem inclusive apresentar melhor estabilidade/viabilidade dos microrganismos em relação aos produtos tradicionais comercializados (Meng et al., 2008). Misturas em pó já são largamente utilizadas em merenda escolar e dietas hospitalares devido à sua praticidade de preparo, menores espaços de estocagem exigidos, estabilidade e longa vida de prateleira. O desenvolvimento de uma mistura em pó probiótica para alimentação em geral possibilitaria a ampliação do acesso a populações até então não consumidoras desse tipo de alimento.

Quando adicionados em produtos alimentícios os probióticos podem perder sua viabilidade, desta forma diversas técnicas de microencapsulação de células têm sido investigadas como uma forma passível de proteção dos probióticos nos produtos. A encapsulação em esferas de hidrocolóides aprisiona ou imobiliza as células dos microrganismos dentro da matriz, o que pode promover proteção nestes meios. Existem

diversos materiais que são empregados para microencapsulação, tais como pectina, κ-carragena, gelatina, amido e gomas, sendo que o mais amplamente utilizado é o alginato de sódio (Stanton et al., 2005).

Com o objetivo de apresentar uma opção de alimento funcional para os consumidores em geral, e principalmente, para aqueles que não apreciam ou não podem consumir derivados do leite, como os intolerantes à lactose, alérgicos à proteína do leite, hipercolesterolêmicos e vegetarianos estritos, o presente trabalho teve por objetivo geral o estudo da produção e encapsulamento de uma linhagem de *L. plantarum* e desenvolver uma mistura em pó com potencial probiótico.

Os objetivos específicos foram:

- Desenvolver tecnologia para produção de microrganismo potencialmente probiótico pelo cultivo de *L. plantarum* BL011 em biorreator submerso testando um resíduo agro-industrial isento de produtos de origem animal;
- Avaliar a produção de biomassa e de ácido láctico por *L. plantarum* BL011;
- Analisar o consumo dos açúcares durante o cultivo em biorreator;
- Microencapsular o *L. plantarum* BL011 em sistema de electrospraying, utilizando alginato de sódio e pectina cítrica como agentes encapsulantes;
- Avaliar a resistência de *L. plantarum* BL011 encapsulado na condição simulada de trânsito gastrointestinal.
- Desenvolver mistura em pó tendo como parâmetro de qualidade a aceitabilidade através de análise sensorial;
- Avaliar a viabilidade de *L. plantarum* BL011 durante a armazenagem em temperatura ambiente em mistura em pó;

- Avaliar a distribuição de *L. plantarum* BL011 no microencapsulado com os suportes testados através de técnicas de microscopia.

Este trabalho está estruturado em capítulos. O Capítulo I apresenta o embasamento teórico pertinente ao desenvolvimento deste estudo. Os capítulos II, III, IV e V estão na forma de artigos científicos submetidos à publicação. Neles estão descritas as metodologias empregadas na condução dos experimentos, bem como a análise e discussão dos resultados. No Capítulo VI são discutidas as considerações finais deste trabalho e as principais conclusões.

CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

1.1 BACTÉRIAS ÁCIDO LÁCTICAS

A expressão bactérias ácido lácticas (LAB) designa-se a um grupo variado de bactérias, as quais possuem como características serem gram-positivos, ácido tolerantes, fastidiosos, destituídos de citocromo, anaeróbios facultativos, não esporulados, catalase negativos, sem mobilidade, e tendo o ácido láctico como principal produto de fermentação (Holzapfel et al., 2001; Savijoki et al., 2006; Mozzi et. al, 2010).

As LAB apresentam viabilidade em pH baixo, além disso possuem um sistema de transporte de ácido láctico e prótons para o exterior celular de forma simultânea, levando a homeostase do pH interno gerando energia (Toro, 2005; Bernardeau et al., 2008). Estas bactérias são classificadas em mesofílicas e termofílicas, conforme sua temperatura de crescimento. As mesofílicas crescem em torno de 30 °C e as termofílicas se desenvolvem a uma temperatura próxima de 42 °C (Sybesma et al., 2006). Os gêneros mais representativos são *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Weissela*, *Carnobacterium* e *Tetragenococcus* (Daniel et al., 2011).

As LAB apresentam exigências nutricionais específicas em relação ao substrato, por meio de metabolismo fermentativo estreitamente sacarolítico. Através dos produtos finais da fermentação podem ser classificadas em dois grupos: as homofermentativas e as heterofermentativas (Figura 1). As primeiras sintetisam apenas ácido láctico, já as heterofermentativas além de ácido láctico, produzem outros compostos como ácido acético, aldeído, diacetil, dióxido de carbono e etanol (Martins et al., 2006; Gonçalves, 2009).

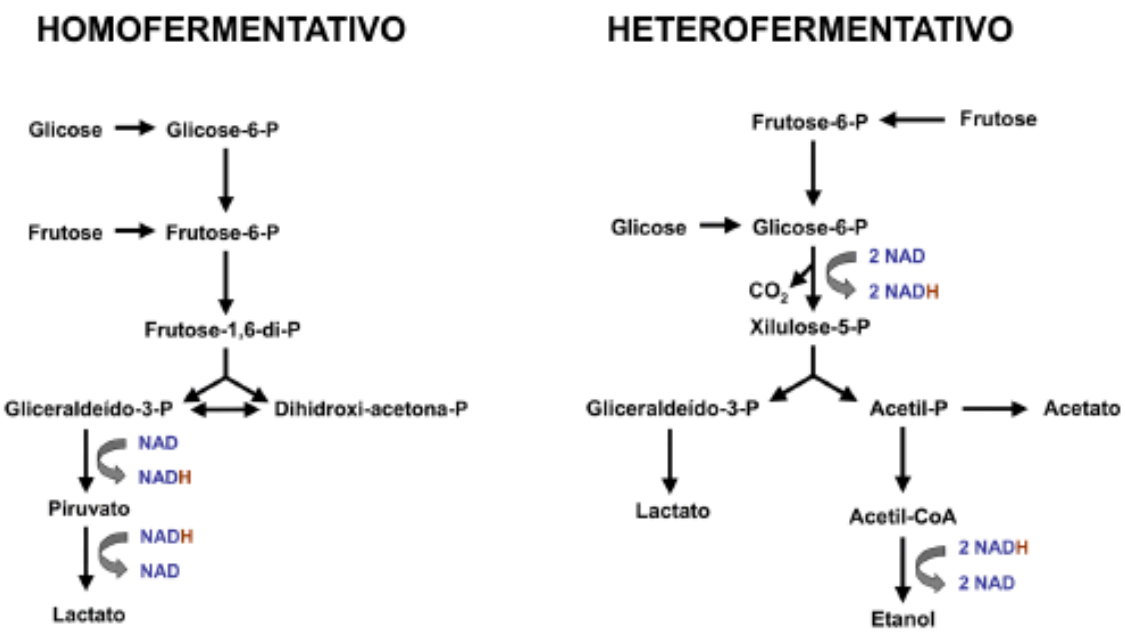

Figura 1: Rota metabólica homo e heterofermentativa de bactérias lácticas do gênero *Lactobacillus* (Adaptado de Costa, 2006).

Por meio da via Embden-Meyerhof (glicólise) as bactérias homofermentativas convertem as hexoses, quase totalmente, em ácido láctico, sendo que um mol de glicose ou outro substrato glicosídico é convertido a dois moles de ácido láctico, na ausência de oxigênio molecular. O processo ocorre em duas etapas: primeiramente a glicose é fosforilada por ATP e clivada para originar gliceraldeído fosfato. Na sequência o gliceraldeído fosfato é transformado em ácido láctico através de várias reações de óxido-redução, acopladas por moléculas de NADH e NAD à fosforização do ADP, ocorrendo desta forma os ciclos de fermentação subsequentes (Brians; Warner, 2003; Gonçalves, 2009). Por meio da lactato-desidrogenase o piruvato é reduzido a ácido láctico, utilizando NADH como doador de hidrogênio. São produzidas quatro moléculas de ATP para cada molécula de glicose fermentada, tendo em vista que duas moléculas são consumidas, a produção líquida é de duas moléculas de ATP por molécula de glicose fermentada (Toro, 2005).

A via pentoses-fosfato (Dickens) é utilizada pelas bactérias heterofermentativas, assim as hexoses são fermentadas a ácido láctico em 50 % e o restante (ácido acético, ácido fórmico, dióxido de carbono, álcool) são simultaneamente fermentados com duas moléculas de ATP e NADH2NAD para que ocorra a continuação do processo de fermentação (Gonçalves, 2009).

As LAB são adicionadas em uma gama de produtos alimentícios para processos fermentativos, bem como incorporadas como probióticos com o objetivo de gerar benefícios à saúde, desta forma devem ser seguras. A LAB do gênero *Lactobacillus* são frequentemente reconhecidas como seguras GRAS (generally recognized as safe – reconhecidamente seguras), tendo em vista a longa história de uso seguro (Donohue, 2004). Porém é necessária uma avaliação mais completa, desta forma, a EFSA (European Food Safety Authority) propôs um método de análise de segurança antes da comercialização de grupos específicos de microrganismos, ocorrendo a elaboração de uma "Presunção de Segurança Qualificada" (QPS), ou seja, avaliar a segurança de um grupo taxonômico definido, um gênero ou grupo de espécies, realizado com base no estabelecimento da identidade, patogenicidade e aplicação final (EC, 2007). Como resultado desta avaliação a EFSA publicou em 2007 que 33 gêneros de *Lactobacillus* possuem QPS, tais gêneros encontram-se na Tabela 1.

Tabela 1: Gêneros de *Lactobacillus* com Presunção de Segurança Qualificada (QPS)

Lactobacillus acidophilus	*Lactobacillus farciminis*	*Lactobacillus paracasei*
Lactobacillus amylolyticus	*Lactobacillus fermentum*	*Lactobacillus paraplantarum*
Lactobacillus amylovorus	*Lactobacillus gallinarum*	*Lactobacillus pentosus*
Lactobacillus alimentarius	*Lactobacillus gasseri*	*Lactobacillus plantarum*
Lactobacillus aviaries	*Lactobacillus helveticus*	*Lactobacillus pontis*
Lactobacillus brevis	*Lactobacillus hilgardii*	*Lactobacillus reuteri*
Lactobacillus buchneri	*Lactobacillus johnsonii*	*Lactobacillus rhamnosus*
Lactobacillus casei	*Lactobacillus kefiranofaciens*	*Lactobacillus sakei*
Lactobacillus crispatus	*Lactobacillus kefiri*	*Lactobacillus salivarius*
Lactobacillus curvatus	*Lactobacillus mucosae*	*Lactobacillus sanfranciscensis*
Lactobacillus delbrueckii	*Lactobacillus panis*	*Lactobacillus zeae*

1.1.1 *Lactobacillus plantarum*

LAB versátil presente em diferentes ambientes, apresenta longa história de uso seguro em alimentos, como o chucrute e as preparações com azeitonas e largamente empregado como probiótico. Dentre muitos estudos, uma mínina parcela sugere que *L. plantarum* esteja envolvido com infecção (De Vries et al., 2006). É um microrganismo membro da microbiota de humanos saudáveis (Castaldo et al., 2009).

1.2 PROBIÓTICOS

Diversas definições de probióticos já foram publicadas, entretanto, a atualmente aceita é que os probióticos são microrganismos vivos que conferem efeito benéfico ao indivíduo, quando administrados em quantidades adequadas (FAO/WHO, 2002). Significando assim que a cultura probiótica deve estar viva e presente em grande quantidade, geralmente valores maiores que 10^6 UFC por mililitro ou miligrama no produto alimentício (Chávez; Ledeboer, 2007; Homayouni et al., 2008; Nualkaekul et al., 2011; Anekella; Orsat, 2013).

De acordo com a legislação brasileira, a quantidade mínima viável para os probióticos deve estar na faixa de 10^8 a 10^9 UFC na porção diária. Valores menores do

que estes podem ser aceitos desde que a empresa comprove a sua eficácia (ANVISA, 2008). Porém a quantidade necessária varia em função da linhagem e do efeito benéfico esperado (Champagne et al., 2005).

Os produtos probióticos disponíveis para uso humano são encontrados em preparações farmacêuticas, na forma líquida ou liofilizada, mas geralmente são vendidos como alimentos lácteos fermentados, principalmente os iogurtes e leites fermentados (Prado et al., 2008).

Membros dos gêneros *Lactobacillus* e *Bifidobacterium* são os principais probióticos reconhecidos. O gênero *Lactobacillus* é muito diverso e envolve um grupo heterogêneo de bastonetes regulares (bacilos), Gram-positivos e não-esporulados, pertencentes ao grupo das LAB, e estão amplamente difundidos na natureza, podendo ser encontrados em plantas e derivados, adubos, alimentos fermentados ou deteriorados; mucosa de humanos e animais como nas cavidades oral, intestino e urogenital (Bernardeau et al., 2008).

Para realizar os benefícios à saúde de seres humanos e animais, os probióticos precisam sobreviver às condições adversas do trato gastrintestinal, especificamente à ação da bile e dos sucos gástrico, pancreático e entérico, desta forma ter condições de permanecer no ecossistema intestinal; não apresentar toxicidade e não ser patogênico; estar viável no período de estocagem do produto, entre outros. As LAB são empregadas na produção de biomassa e de metabólitos, a produção de biomassa era destinada para utilização como culturas iniciadoras em alimentos fermentados. No entanto, em meados dos anos 80 iniciou-se o interesse na otimização da produção de biomassa de LAB. Comumente o critério para a seleção de culturas iniciadoras envolve taxa de acidificação e a produção de *flavors*. A utilização de substratos agro-industriais de baixo

custo é uma importante alternativa para otimizar a produção de biomassa (Teusink; Smid, 2006).

Os microrganismos potencialmente probióticos (Tabela 2) apresentam alguns critérios de seleção, os quais estão mensionados abaixo (Champagne et al., 2005; Shah; 2007; Prado et al., 2008; Azizpour et al., 2009; Rivera-Espinoza; Gallardo-Navarro, 2010):

- Propriedades tecnológicas: para a indústria, o probiótico precisa atingir significativo rendimento de biomassa, facilidade de concentração, manter-se viável após processos tecnológicos (congelamento e secagem). Também deve possuir baixo custo, ser estável no período de estocagem e apresentar facilidade para incorporação em alimentos;

- Propriedades de adesão: os probióticos devem apresentar boa adesão na superfície da mucosa intestinal, para que permaneça por mais tempo e não seja eliminado pelo peristaltismo, e assim conseguir desempenhar suas funções como a competição com microrganismos patogênicos. Porém para que os benefícios sejam alcançados e para que os probióticos permaneçam no cólon, é necessário que se tenha um consumo regular de alimentos fonte de probióticos.

- Critérios de segurança: o probiótico deve ser seguro, não carcinogênico, não patogênico, ser sensível a antibióticos bem como não transmitir genes de resistência a esses compostos e não ser invasivo.

- Propriedades antimicrobianas: as culturas probióticas devem competir por nutrientes com microrganismos patógenos e sintetisar compostos inibitórios, como peróxido de hidrogênio, bacteriocinas e ácidos orgânicos.

- Propriedades biológicas: o probiótico precisa sobreviver no produto e no trato gastrintestinal, especificamente à acidez do estômago e os sais biliares.

Inúmeras pesquisas vêem sendo realizadas sinalizando os efeitos benéficos dos probióticos no hospedeiro, sendo eles, diminuição das infecções gastrintestinais; redução no colesterol sérico; atividade antimicrobiana; estimulação do sistema imune; auxilia no metabolismo da lactose; propriedades antimutagênicas, anticarcinogênicas e antidiarréicas; melhoria nos sintomas da síndrome do intestino irritável; supressão de infecções ocasionadas por *Helicobacter pylori*; e redução da obesidade e da dermatite atópica (Lee et al., 2008; Azizpour et al., 2009; Saad et al., 2013; Palacios et al., 2014). No entanto é imprescindível ter o conhecimento que os efeitos benéficos à saúde proporcionados pelos probióticos, são específicos para cada linhagem, sendo assim, nenhuma linhagem é capaz de prover todos os benefícios citados, onde nem mesmo linhagens da mesma espécie são eficazes contra condições de saúde específicas (Shah, 2007).

Tabela 2 - Microrganismos utilizados como probióticos.

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Outros**
L. acidophilus	*B. adolescentes*	*Streptococcus cremoris*
L. casei	*B. animalis*	*Streptococcus thermophilus*
L. delbrueckii ssp. *bulgaricus*	*B. bifidum*	*Streptococcus intermedius*
L. brevis	*B. longum*	*Streptococcus diacetylactis*
L. cellobiosus	*B. infantis*	*Lactococcus lactis*
L. curvatus	*B. thermophilum*	*Saccharomyces cerevisiae*
L. fermentum	*B. breve*	*Saccharomyces boulardii*
L. paracasei	*B. lactis*	*Propionibacterium reundenreichii*
L. plantarum	*B. essensis*	*Enterococcus faecium*
L. reuteri	*B. laterosporus*	*Enterococcus faecalis*
L. johnsonii		*Leuconostoc mesenteroides*
L. rhamnosus		*Pediococcus acidilactici*
L. gasseri		*Sporolactobacillus inulinus*
L. crispatus		*Bacillus cereus* var. *toyoi*
L. helveticus		*E. coli* Nissle
L. amylovorus		
L. gallinarum		

Fonte: Azizpour et al., 2009; Saad et al., 2013.

1.3 FERMENTAÇÕES PARA PRODUÇÃO DE BACTÉRIAS LÁCTICAS EM BIORREATOR

Os bioprocessos realizados por microrganismos, tradicionalmente denominados como processos fermentativos, são importantes fontes de produtos biológicos empregados nas indústrias química, farmacêutica e de alimentos. Vem se observando, ao longo dos anos um aumento significativo na quantidade de bioprodutos comerciais, especificamente metabólitos secundários. São observadas também importantes modificações na configuração de biorreatores, para melhorar o desempenho e garantir operações mais seguras (Pereira; Bom; Ferrara, 2008).

Os cultivos em batelada são geralmente utilizados para definir parâmetros de processo e fisiológicos, sendo importantes para a criação de modelos cinéticos (Youssef; Goma; Olmos-Dichara, 2005). Estes cultivos possibilitam obter pouco substrato residual e alta concentração de produto, como ácido láctico, principal metabólito das LAB (Ghaly et al., 2004).

O cultivo em batelada possui algumas desvantagens, tais como a produção de ácido láctico durante a fermentação interferindo no crescimento celular, podendo inibir a produção de biomassa. Altas concentrações da fonte de carbono produzem maiores concentrações de ácido láctico, porém sabe-se que elevadas concentrações de fonte de carbono e de nitrogênio inibem o crescimento dos microrganismos (Coelho et al., 2011).

A fermentação por batelada alimentada possibilita a utilização de limitações do crescimento celular, demonstrando alta concentração de biomassa em LAB, eficiente conversão de lactose em ácido láctico e baixas concentrações de substrato residual (Aguirre-Ezkauriatza et al., 2010; Elmarzugi et al., 2010; Hwang et al., 2011). A batelada alimentada não apresenta saída de produto do reator, o substrato é adicionado para controlar a velocidade da reação, possibilitando alcançar alta densidade celular,

controlando a inibição do crescimento e formação de produto, pelo monitoramento da velocidade de crescimento celular (Aguirre-Ezkauriatza et al., 2010; Elmarzugi et al., 2010; Hwang et al., 2011; Radwan; Moussa; Alsarra, 2011).

Apesar das vantagens, o cultivo em batelada alimentada representa uma operação complexa, com necessidade de controladores computadorizados sofisticados para biorreator. Além disso, para as LAB é fundamental ter um profundo conhecimento da fisiologia celular para evitar a desestabilização do regime de alimentação, causada pelo excesso de acidificação e desequilíbrio no suprimento de oxigênio (Aguirre-Ezkauriatza et al., 2010; Elmarzugi et al., 2010; Hwang et al., 2011; Radwan; Moussa; Alsarra, 2011).

Com relação ao cultivo contínuo, o biorreator é operado em estado estacionário e a produção de biomassa ocorre em estado fisiológico controlado, por meio da manipulação de parâmetros ambientais, como a taxa de diluição e a determinação da taxa de crescimento específica de LAB (Doleyres et al., 2002).

Este cultivo possui como principal desvantagem o fato de que, a concentração de células e dos produtos de interesse são muito diluídos. Pode-se minimizar este inconveniente utilizando a reciclagem de células no biorreator, com aumento da recuperação das células e do ácido láctico (Wee; Ryu, 2009).

A reciclagem das células pode ser realizada por meio de um sistema de membrana de ultra ou microfiltração, onde as moléculas menores (ácido láctico) ultrapassam os poros da membrana, sendo assim removidas, não causando inibição do crescimento celular. As células por sua vez, permanecem na membrana, levando a um aumento na produtividade (Corre; Madec; Boyaval, 1992; Lacroix; Yildirim, 2007). Contudo, o cultivo contínuo de LAB é pouco empregado na indústria devido à alta

susceptibilidade de contaminação e perda da viabilidade celular (Lacroix; Yildirim, 2007).

Todas as formas de operação do biorreator (batelada, batelada alimentada, contínuo) apresentam vantagens e desvantagens, desta forma, a escolha do melhor processo vai depender das propriedades cinéticas de cada microrganismo, do substrato empregado e de aspectos econômicos do processo.

Para a obtenção de um processo biotecnológico satisfatório, também é preciso cultivar as células microbianas em meio de cultivo adequado, devendo atender todas as necessidades nutritivas do microrganismo, para a produção de biomassa e metabólitos de interesse industrial. As LAB são fastidiosas e exigem meios de cultivo complexos com alto valor nutricional, contendo carboidratos, minerais, vitaminas e especialmente, aminoácidos, proteínas ou proteínas hidrolisadas (Vásquez; Murado, 2008). Estas necessidades de nutrientes também ocorrem devido à habilidade biossintética limitada de sintetizar vitaminas do complexo B e aminoácidos, pelos microrganismos (Hofvendahl; Hahnhägerdal, 2000).

O meio de cultivo mais utilizado para cultivar e isolar bactérias lácticas é o meio MRS (De Man; Rogosa; Sharpe, 1960), o qual é rico em vitaminas, aminoácidos, peptídeos, sais minerais e açúcar. No entanto, para aplicação em larga escala, é inviável devido ao custo elevado, desta forma, justifica-se o uso de fontes de carbono e nitrogênio alternativas (Victorelli, 2011).

Como fontes alternativas pode-se citar os resíduos agro-indutriais, que são produzidos em grande quantidade, e potenciais fontes de carboidratos para fermentação, de baixo custo de aquisição em comparação aos meios de cultivo comerciais, tornando-os opções muito interessantes (Zhang et al., 2007).

Os resíduos agro-industriais mais comumente utilizados na produção de LAB são materiais lignocelulósicos em geral (Yáñez et al., 2003; Wee; Ryu, 2009; Abdel-Rahman; Tashiroc; Sonomoto, 2011), soro de leite, permeado de soro (Brinques; Peralba; Ayub, 2010; Altiok; Tokatli; Harsa, 2006; Mondragón-Parada et al., 2006), vísceras de peixe (Horn; Aspmo; Eijsink, 2005) e milhocina (Li et al., 2010).

Dentro deste contexto, tem-se o soro ácido de soja, um efluente obtido da produção de proteína isolada de soja (PIS). Uma das empresas geradoras deste resíduo é a Dupont localizada na cidade de Esteio (RS), este efluente especificamente, apresenta composição nutricional satisfatória para produção de LAB.

Para a obtenção do produto de interesse (PIS) a empresa produz aproximadamente 28 m^3 de efluente para cada tonelada de PIS, sendo uma produção mensal de aproximadamente 50.000 m^3. A empresa possui uma estação de tratamento do resíduo, a qual tem uma despesa mensal em torno de R$ 175.000,00 (nos valores de meados de 2013) com produtos químicos e materiais de laboratório, além de outros gastos, para dar o destino correto ao resíduo produzido. Este estudo é a primeira pesquisa que utiliza este resíduo agro-industrial para a produção de *L. plantarum*.

1.4 MICROENCAPSULAÇÃO

A microencapsulação conceitua-se como uma tecnologia de recobrimento de partículas de material líquido ou gasoso, formando cápsulas muito pequenas que liberam seu conteúdo de forma controlada e/ou em condições específicas (Fávaro-Trindade et al., 2008; Mirzaei et al., 2012).

A técnica de microencapsulação vem sendo empregada na área de alimentos objetivando proteger substâncias sensíveis à luz, oxigênio, tempo e temperatura de armazenamento, impossibilitando interações entre compostos, tornando o produto mais

estável e assim, prolongando sua vida de prateleira (Desai; Park, 2005). Os materiais que estão sendo protegidos pela microencapsulação envolvem aqueles utilizados na indústria de alimentos, como os aromatizantes, minerais, corantes, enzimas, vitaminas, aminoácidos, hidrolisados proteicos, ácidos, além dos microrganismos (Fávaro-Trindade et al., 2008).

Possibilitar a liberação controlada do material ativo é também um objetivo da microencapsulação (Anal; Singh, 2007). Porém, esta liberação modifica-se conforme a origem do agente encapsulante e geralmente acontece pelos mecanismos de dissolução em solventes, ação de força osmótica, permeabilidade seletiva, mudanças de temperatura e pH, ruptura mecânica, ação de enzimas e difusão (Gouin, 2004).

Diferentes técnicas de microencapsulação estão sendo utilizadas na área de alimentos, onde a escolha da técnica é dependente da aplicação que a microcápsula terá, do tamanho requerido, do mecanismo de liberação e das propriedades físico-químicas, do material ativo e do agente encapsulante (Fávaro-Trindade et al., 2008).

Os microencapsulantes comumente utilizados são semipermeáveis, com morfologia esférica, recoberta por uma membrana resistente sólida ou sólida/líquida, com um diâmetro de mícrons a 1 mm (Anal; Singh, 2007). Os agentes encapsulantes geralmente empregados na microencapsulação são alginato, quitosana, carboximetilcelulose (CMC), gelatina, carragena, e pectina (Li et al., 2009; Burey et al., 2009).

1.4.1 Electrospraying

Caracteriza-se por um método de pulverização de líquido (electro pulverização), através de forças elétricas (Figura 2). O tamanho das gotículas de electrospray pode variar desde centenas de micrômetros até várias dezenas de nanômetros, a distribuição

do tamanho das gotículas é monodispersa. A formação das esferas e o tamanho podem ser controlados, por meio da taxa de fluxo do líquido e a tensão no bocal do capilar. Devido as esferas serem eletricamente carregadas o controle do seu movimento pelo campo elétrico é facilitado (Jaworek; Sobczyk, 2008).

A técnica de electrospraying pode ser amplamente aplicada em ambos os processos, industriais e instrumentações científicas, as pesquisas de electro-microencapsulação e electro-emulsificação estão sendo utilizadas no desenvolvimento de medicamentos, de novos ingredientes funcionais, podendo ser utilizadas matrizes de encapsulamento como proteínas e polissacarídeos que representam biopolímeros naturais, desempenhando um papel essencial na estabilização das formulações de alimentos (Jaworek; Sobczyk, 2008; López-Rubio et al., 2012).

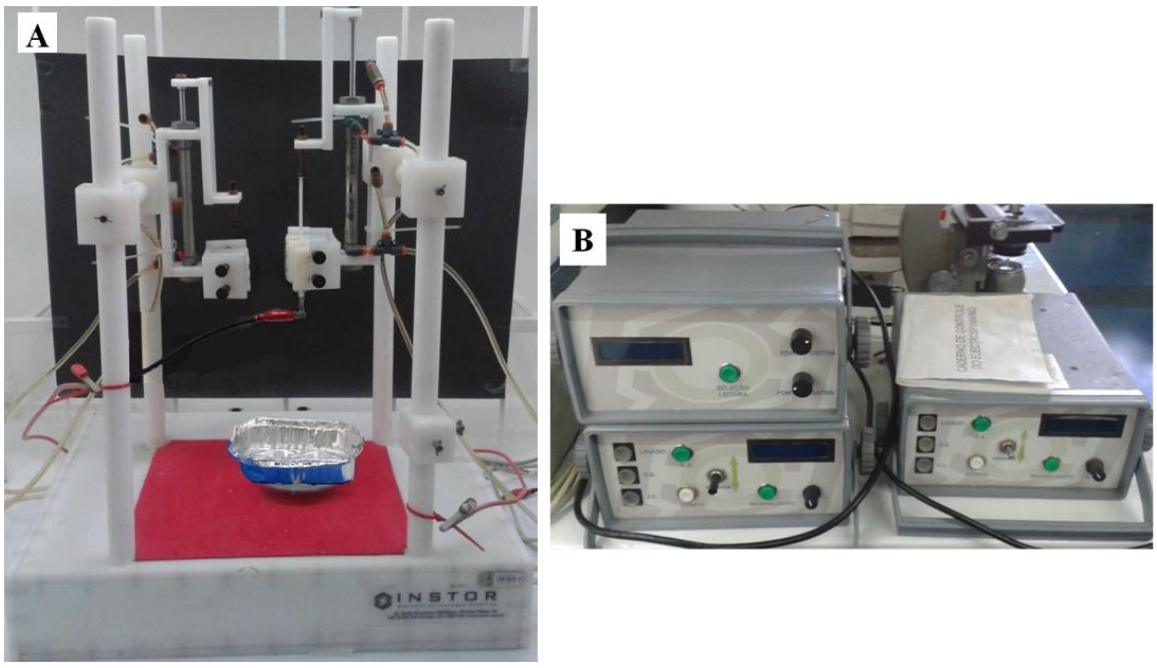

Figura 2 - Esquema do equipamento de electrospinning/electrospraying, em (A) a estrutura de elaboração das microcápsulas e em (B) as unidades de controle (Fonte: o autor).

1.4.2 Alginato

São polímeros heterogêneos com ampla faixa de composições químicas, tamanho molecular e propriedades funcionais. Representam um grupo de polissacarídeos não ramificados compostos por resíduos de ácido-β-D-manurônico (M) e ácido-α-Lgulurônico (G) ligados por ligações do tipo (1→4). Estes componentes podem ser constituídos de díades de manuronato (MM), díades de guluronato (GG) e hetero-díades (MG/GM) e a quantidade de cada díade modifica-se conforme a fonte de alginato (Heng, et al., 2003; Simpson, et al., 2003).

O alginato é um polieletrólito carregado negativamente a pH neutro ou básico. Com um grupo funcional carboxílico em todas as unidades de M e G. A ligação cruzada de cadeias de alginato com cátions divalentes, tais como Ca^{2+}, Zn^{2+} e Cu^{2+}, leva a obtenção de um gel, formando uma rede tridimensional (Heng et al., 2003; Simpson et al., 2003).

1.4.3 Pectina

Polissacarídeos complexos obtidos da parede celular das plantas, constituído por um esqueleto maior que 100 monômeros de ácido galacturônico unidos por meio de ligações α - (1→4), que são em parte metilesterificados (Pérez et al., 2000).

O grau de esterificação vai definir as propriedades das pectinas, sendo que as pectinas nativas são de alto grau de metoxilação (HM) (> 50 % esterificadas) e as pectinas de baixo grau de metoxilação (LM) (< 50 % esterificadas) são frequentemente formadas pela de-esterificação ácida controlada das pectinas HM. A de-esterificação pelo uso de tratamentos com amônia origina um tipo diferenciado de pectina, as pectinas LM aminadas, estas têm capacidade de fazer ligações cruzadas com íons divalentes como os íons cálcio. Nesta ocorre a junção de zonas pela sua ordenação lado-

a-lado das cadeias de pectina onde sequências específicas de monômeros de ácido galacturônico formam cavidades onde os íons cálcio encaixam-se. O resultado das ligações cruzadas é a gelatinização (Braccini; Pérez, 2001; Tho et al., 2005).

1.5 PRODUTO ALIMENTÍCIO VEÍCULO DE PROBIÓTICO

O alimento veículo dos probióticos é considerado um dos fatores mais importantes na regulação da colonização destes microrganismos no trato gastrointestinal devido aos efeitos sinérgicos potenciais desempenhados pela matriz alimentícia (Ranadheera et al., 2010). Contudo, a maioria destes produtos apresenta uma desvantagem que é a necessidade da manutenção da cadeia do frio até o consumidor final, dificultando dessa forma a maior inserção de alimentos suplementados com probióticos a toda a população.

Os alimentos lácteos fermentados representam a maior parte dos alimentos contendo culturas probióticas (Céspedes et al., 2013). Porém diferentes alimentos vêm sendo utilizados como veículos de probióticos como: barras de cereais (Chen; Mustapha, 2012), chocolate (Possemiers et al., 2010); produtos de soja (Singh et al., 2011); chucrute (Yu et al., 2012); picles (Çetin, 2011); bebidas preparadas a partir de cereais (Charalampopoulos; Pandiella, 2010); e produtos a base de frutas e legumes, sendo eles vegetais fermentados (Lee et al., 2011), frutas minimamente processadas (Alegre et al., 2011; Rößle et al., 2010) e azeitonas de mesa (Hurtado et al., 2012).

A elaboração de produtos probióticos não lácteos possibilita o consumo destas culturas benéficas por pessoas intolerantes à lactose, alérgicas às proteínas do leite, hipercolesterolêmicas, vegetarianas estritas ou aqueles que residem em locais onde não possuem acesso aos produtos lácteos (Rivera-Espinoza; Gallardo-Navarro, 2010).

1.5.1 Análise sensorial

Pode ser conceituada como uma ferramenta moderna empregada para a criação de novos produtos, reformulação dos produtos já comercializados, estudos de vida de prateleira (*shelf life*), definição das diferenças e semelhanças encontradas em produtos concorrentes, identificação da preferência do consumidor por um produto e para a otimização e melhoria da qualidade (Slongo, 2008).

A análise sensorial possui função importante na avaliação das propriedades sensoriais de alimentos (cor, aparência, textura, sabor, aroma), relacionados com a composição química, processamento, além do tipo de embalagem e armazenamento (Slongo, 2008).

O consumidor é quem determina a qualidade de um produto alimentício, através da análise sensorial. Avaliações sensoriais originam resultados que possibilitam compreender as transformações estudadas, e analisar seu impacto na preferência, atitude e expectativa do consumidor sobre o produto (Slongo, 2008).

A escala hedônica é um dos métodos mais utilizados na aceitabilidade e preferência de um alimento, onde o julgador e/ou o consumidor avalia o produto seguindo uma escala previamente estabelecida através de atributos como “gostei” e “desgostei” (Chaves; Sprosser, 2001).

INTRODUÇÃO AOS CAPÍTULOS II, III, IV E V

Os Capítulos II, III, IV e V estão apresentados na forma de artigos científicos. Em cada um destes capítulos consta a introdução, os materiais e as metodologias empregadas, juntamente com a discussão dos resultados obtidos e as conclusões pertinentes a cada etapa de trabalho desenvolvida.

O primeiro artigo (Capítulo II - "Technologies for probiotics production and alternative encapsulation methodologies to improve their resistance under adverse environmental conditions") é uma revisão que aborda três tópicos principais: Tecnologias de fermentação para a produção de microorganismos potencialmente probióticos; Formulações de meio para o cultivo de LAB e produção de ácido láctico - Meios alternativos de cultivo para a produção de microorganismos potencialmente probióticos; e Microencapsulação de microorganismos potencialmente probióticos. Este artigo foi submetido ao periódico Food Reviews International.

O segundo artigo (Capítulo III – "*Lactobacillus plantarum* BL011 cultivations in industrial isolated soybean protein acid residue") foi conduzido com o objetivo de produzir células com potencial probiótico em biorreator submerso, utilizando meio de cultivo vegetal, definindo parâmetros de processo. Este artigo foi submetido ao periódico Brazilian Journal of Microbiology.

No terceiro artigo (Capítulo IV – "Electrospraying microencapsulation of *Lactobacillus plantarum* enhances cell viability under refrigeration storage and simulated gastric and intestinal fluids") foi realizada a microencapsulação do microrganismo produzido em meio vegetal, através da técnica de electrospraying, além de submetê-lo aos sucos gástrico e intestinal e realizar a caracterização das cápsulas e dos suportes utilizados na microencapsulação. Este artigo foi submetido ao periódico Journal of Functional Foods.

No quarto artigo (Capítulo V – "Viability and alternative uses of a dried powder, microencapsulated *Lactobacillus plantarum* without the use of cold chain or dairy products") foi obtido o pó potencialmente probiótico, onde, após a microencapsulação o microrganismo foi submetido a liofilização, em seguida foi avaliada a estabilidade em temperatura ambiente, sucos gástrico e intestinal, e a análise sensorial. Este artigo foi submetido ao periódico Food & Function.

CAPÍTULO II – TECHNOLOGIES FOR PROBIOTICS PRODUCTION AND ALTERNATIVE ENCAPSULATION METHODOLOGIES TO IMPROVE THEIR RESISTANCE UNDER ADVERSE ENVIRONMENTAL CONDITIONS

Este artigo foi submetido ao periódico ***Food Reviews International***

Technologies for probiotics production and alternative encapsulation methodologies to improve their resistance under adverse environmental conditions

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Abstract

Probiotics are dietary supplements of live microorganisms producing beneficial health effects on the host by improving intestinal balance and nutrient absorption. Among the probiotic microorganisms, those classified as lactic acid bacteria (LAB) are of major importance to food and feed industries. Fermentation processes are the fundamental step in order to obtain biological products for the pharmaceutical, chemical, and food industries. Probiotics can be produced using alternative carbon and nitrogen sources, such as agro-industrial residues, at the same time contributing to reduce process costs. On the other hand, the probiotics survival in formulated food products, as well as in the host gut, is essential from the nutritional aspect and health benefits. Therefore, several cell microencapsulation techniques have been investigated as a way to improve cell viability and survival under adverse environmental conditions, such as the gastrointestinal milieu of host. In this review, we discuss different aspects of probiotics production technologies, including the formulation of culture media, and some aspects of cell microencapsulation techniques required to improve their survival in the host.

Keywords Probiotics, lactic acid bacteria, fermentation technologies, bioproducts, cell microencapsulation.

Introduction

Food research and product development in the area of functional foods have been motivated by the increasing life expectancy, health concerns, and the improvement of quality of life of the general population. Bioactive components of fermented foods and probiotics are recognized as beneficial ingredients because of their long history of safe use.[1] In this sense, probiotics are defined as live microorganisms that, when used in adequate amounts, confer beneficial effects on the health of the host.[2]

Several studies show the beneficial effects of probiotics for the host. These health benefits include decreased gastrointestinal infections, reductions in serum cholesterol, antimicrobial activities (for example, suppression of infections caused by *Helicobacter pylori*), stimulation of the immune system, improved lactose metabolism, antimutagenic and anticarcinogenic effects, reduced symptoms of irritable bowel syndrome, and atopic dermatitis.[3-6]

Several microorganisms have been used as probiotics. Among the most commonly cited in the scientific literature and commercially used, are strains of *Lactobacillus rhamnosus, L. acidophilus strains, L. casei, L. plantarum, L. reuteri*, *Streptococcus thermophilus, Enterococcus faecium, Bifidobacterium bifidum, B. brief, B. lactis, and B. longum.* [7]

Reports on the literature for the production of probiotics describe batch, fed-batch, and continuous cultures, using different types of bioreactors and feeding strategies.[8-12] Some authors have also attempted the use of immobilized-cells and membrane system bioreactors aiming at improving biomass production.[13-15]

Complex media, which is generally used for the growth of probiotic bacteria, are not economically sound because of their expensive component nutrients, such as yeast extract, peptone, and salts, impacting the cost of fermentation media that ends up

representing up to 30 % of the total costs of the microbial production.[16] Alternatively, probiotic bacteria can be produced using cheap substrates such as agro-residues, which are used as media components for cultivation.[17] Some agro-residues reported as nutrients in microbial cultures are cassava bagasse, sugarcane bagasse, sugar beet pulp, coffee husk and pulp, apple pomace, oilcakes, wheat/rice bran, among others. Their uses have been described to obtain high value-added bio-products, such as enzymes, organic acids, ethanol, amino acids, aroma, and unicellular proteins.[18]

One of the main concerns regarding the use of probiotic bacteria in food products is their relative feeble resistance towards adverse conditions, such as environmental pH and temperature. Cell viability must be kept in the products throughout the shelf life time and during consumption, and numerous techniques have been attempted in trying to improve this aspect.[13, 19, 20] Among the many promising technologies that have been reported, the immobilization of cells has shown to be exceptionally interesting, allowing increased cell resistance and survival. Of different immobilization techniques, the most successful are those based on emulsification,[21, 22] or on extrusion.[20, 23]

In this context, the aim of this review is to discuss some important production technologies for probiotic bacteria, especially looking at cost-effective alternatives, and at some microencapsulation techniques that could prove useful in extending cell viability in formulated products.

Fermentation technologies for the production of potentially probiotic microorganisms

The fermentation process is the key step to obtain microbial biomass and biological products used by the pharmaceutical, chemical, and food industries.[24] In the last decade, there was a significant increase in the number of commercial bioproducts, in

special therapeutic proteins and secondary metabolites produced by recombinant DNA technology. In parallel with this use of molecular biology, it has also been noticed some significant advances in bioreactor engineering aiming to improve their performances and ensuring safer industrial operations.[25]

Batch fermentations are frequently used to determine various operating and physiological parameters that required for the development of kinetic models for both transient and steady state cell cultivations.[26] In the case of probiotics productions, batch fermentations allow us to obtain low residual substrate and high product concentrations, especially of lactic acid, which is one of the most important metabolites of potential probiotic bacteria. However, batch fermentations lack the efficiency of other operational cell cultivation systems such as fed-batch and continuous cultures, in which the maximal cell growth can be controlled. Additionally, when biomass is the desired product, excessive formation of lactic acid and other organic acids might be a problem for cell propagation.[10]

Therefore, the application of cell growth limitation as it is possible in fed-batch cultivations, showed good results in terms of LAB biomass production, high conversions of lactose into lactic and other organic acids, low concentrations of residual substrate, and increased cell viability of lyophilized preparations.[27-29] Fed-batch cultivations produce no outputs from the reactor during the fermentation. However, one substrate is added in order to control the reaction rate, allowing reaching high cell densities, while controlling growth inhibition and/or product formation by cell growth speed control. Nevertheless, fed-batch remains a complex operation that requires sophisticated computer-to-bioreactor controls. Additionally, in the case of LAB and other potentially probiotic bacteria, in-depth cell physiology knowledge is necessary to

avoid destabilization of the feed regime caused by excessive acidification and imbalances in oxygen supply.[27, 30]

Concerning the use of continuous cultures, few studies are found in the literature for the production of potentially probiotic microorganisms. Continuous cultivation is characterized by steady state bioreactor operation and the production of cells develops under controlled physiological state by manipulating environmental parameters, such as the dilution rate (D, h^{-1}) and the determination of the specific growth rate of LAB in use.[31] One of the main problems of continuous cultures is the diluted concentration of cells and products that are obtained. This problem might be overcome by recycling cells to the bioreactor, increasing final cell recovery and lactic acid, which was shown to increase by 60 % in this system when compared to batch operations.[17] Cell recycle is achieved using membrane systems, in which cells are kept in the bioreactor by ultra- or microfiltration membranes. Small molecules, such as lactic acid, diffuse through the membrane pores according to their size, thus inhibitory metabolic products are eliminated in the permeate, whereas cells are concentrated, increasing productivities.[15, 32] For instance, high cell yield, representing a 15-fold increase of productivity compared to batch systems, was obtained in continuous cultures of *B. bifidum* in a stirred-tank bioreactor coupled to an ultra filtration device.[15]

Continuous cultures of LAB with cell-recycle membrane systems have shown good results concerning increased lactic acid productivity (separated in the permeate), among other important metabolites.[33, 34] However, in some cases, there were reports of cells undergoing induced stress, resulting in changes in the physiology and overall decrease of cell viability.[35, 36]

Continuous cultures of LAB enable the production of cells bearing different physiological states and special controlled stress conditions, such as those found in the

digestive tract of hosts. For instance, it has been demonstrated that a two-phases continuous culture produces potentially probiotic microorganisms stress-adjusted, providing a new and efficient alternative for screening sub lethal stresses as compared to conventional batch cultures.[32]

Despite these advantages, the continuous cultivation of LAB remains a difficult operation to be performed at industrial level, because of the high contamination susceptibility and loss of cell viability of this group of bacteria.[32] Different systems of cell cultivation, LAB, and media for the production of these probiotic or potentially probiotic bacteria are shown in Table 1.

Table 1. Different approaches of cell cultivation and media formulation for the production of LAB considered being probiotic.

Strain	Production/productivity of biomass or lactic acid	Media	Fermentation technologies	Reference
L. rhamnosus NBRC 3863	6.6 g l^{-1} biomass	Hydrolyzed fish waste	Batch fermentation	(91)
L. plantarum VTT E-79098	$8 \cdot 10^9$ log CFU ml^{-1} biomass	Malt sprout	Batch fermentation	(92)
L. paracasei	31.5 g $l^{-1}h^{-1}$ lactic acid	Peptone, yeast extract, glucose	Continuous cell-recycle membrane	(93)
L. rhamnosus ATCC 10863	125 g l^{-1} lactic acid	Soybean hydrolysate	Batch fermentation	(94)
L. casei LA-04-1	210 g l^{-1} lactic acid	Glucose, yeast extract, soya peptone, corn steep liquor	Pulse fed-batch, constant feed rate fed-batch, constant residual glucose concentration fed-batch, exponential fed-batch	(95)
L. delbrueckii NRRL B-445*; L. Plantarum sp* 14431	34.5 and 42.8 g l^{-1} lactic acid	Alfalfa fiber, soy fiber, corncob, wheat straw	Batch fermentation	(96)
L. intermedius NRRL B-3693	17 g l^{-1} biomass	Corn steep liquor	Fed-batch fermentation; continuous cell-recycle fermentation	(97)
L. plantarum NCIMB 8826	9.16 log CFU ml^{-1}	White flour, oat flour and bran	Batch fermentation	(98)
L. casei NRRL B-441	2.5 g dm^{-3} h^{-1} lactic acid	Whey	Batch fermentation	(43)
L. casei KH-1	5.380 g l^{-1} biomass	Corn steep liquor	Batch fermentation	(99)
Lactococcus lactis subsp. *Lactis*	18.9 g l^{-1} lactic acid	Potato starch waste	Fed batch fermentation	(37)
L. rhamnosus NBRC 3863	1.68 g l^{-1} lactic acid	Rice bran	Batch fermentation	(100)
L. casei strain 01	50 g l^{-1} lactic acid	Malt combing nuts and whey permeate	Batch fermentation	(101)
L. amylophilus GV6	46 g of lactic acid from 100 g of wheat bran	Wheat bran, red lentil flour	Solid state fermentation	(102)
L. bulgaricus	40 g l^{-1} biomass	Cheese whey permeate	Membrane recycle bioreactor	(103)
L. casei BPG4	$2 \cdot 10^{10}$ cell g^{-1} of freeze-dried product; 2.7 g l^{-1} biomass	Goat milk whey	Batch, continuous and fed-batch fermentation	(27)
L. casei NCBI AF526388	3 g l^{-1} biomass	Whey	Batch fermentation	(44)
*L. lactis*447 and *L. casei*, subsp. *rhamnosus*1753	55 g l^{-1} biomass; 30 g $l^{-1}h^{-1}$ lactic acid	Lignocellulosic hydrolysate of crushed corn cobs	Membrane recycle bioreactor	(104)
Enterococcus mundtii QU 25	119 g l^{-1} lactic acid	Cellobiose	Batch fermentation	(105b)
L. brevis (S3F4) and *L. plantrum* (XS1T3-4)	39.1 g l^{-1} lactic acid	Corncob	Batch fermentation	(106)
L. bifermentans DSM 20003	62.8 g l^{-1} lactic acid	Wheat bran hydrolysate	Batch with cell Immobilization	(107)
L. rhamnosus ATCC 9595 (CECT288)	32.5 g l^{-1} lactic acid	Apple pomace	Batch fermentation	(108)
L. lactis CECT-4434	10.8 g l^{-1} lactic acid	Trimming vine shoots; distilled wine lees (vinasses)	Batch SSF	(109)
L. lactis IO-1	10.9 g l^{-1} lactic acid	Sugar cane baggase	Batch fermentation	(110)
L. plantarum LB/103-1-5	9.44 log CFU ml^{-1} biomass	Aloe vera juice	Batch fermentation	(111)
*L. paracasei*LA104	122.91 g l^{-1} lactic acid	Fresh sweet potato	Batch SSF	(112)

L. rhamnosus.	10 g l^{-1} lactic acid	Sweet potato by-product	Batch fermentation	(113)
Bifidobacterium animalis subsp. *Lactis* Bb-12	13.1×10^{10} CFU $l^{-1}\cdot h$	Cabbage juices fresh	Batch fermentation	(114)
L. plantarum NCIMB 8826	102.3 g l^{-1} lactic acid	Barley extract	Batch SSF (simultaneous saccharification and fermentation)	(115)
L. delbrueckii	25 g l^{-1} lactic acid	Municipal solid organic waste	Fed batch fermentation	(116)
L. casei	0.61 g $l^{-1}h^{-1}$ lactic acid	Soybean straw hydrolysate	Batch fermentation	(117)
L. rhamnosus ATCC 7469; *L. fermentum* PL-1	0.52 g $l^{-1}h^{-1}$; 0.32 g $l^{-1}h^{-1}$	Brewer's spent grain hydrolysate	Batch fermentation	(118)

Media formulation for LAB cultivation and lactic acid production

Alternative cultivation media for the production of potentially probiotic microorganisms

Many food and agro-based industries produce large volumes of solid and liquid wastes that are often discharged in the environment, or are sub-utilized in land farming, presenting potential ecological hazards. Correct utilization, recycling, and reprocessing of these wastes would therefore be beneficial to the environment, and would possibly add some economical advantage to industries.[37] Several parameters interfere with the use of wastes and agro-residues in bioprocess, among them, the type (organic, inorganic) and availability of the nitrogen and carbon sources, the fermentation process (submerged or solid-state), the temperature, and pH.[16, 38] The heterogeneous characteristics of the physical and chemical nature of wastes and agro-residues limit the development of bioconversion processes based on them. In special, the bioconversion of the most abundant starchy and lignocellulosic materials by LAB is still very problematic from the bioprocess point of view.[38]

Microorganisms play an important role in waste utilization because of their ability to degrade or convert these organic materials, and LAB can produce lactic acid, among other organic acids, using some of such wastes as substrates.[39] The industrial application of microorganisms that produce lactic acid from starch-containing wastes eliminates the need for saccharification and/or liquefaction, resulting in decreased production costs. For instance, in recent years research has been focused towards the development of economically effective and sustainable biological and biotechnological approaches to produce lactic acid on an industrial scale using industrial residues, in contraposition to the traditional use of pure glucose, which is expensive.[17, 37]

Considering the bioprocess to obtain LAB and their main product, lactic acid, a key issue is always the cost of medium. The use of some agro-residues to obtain biotechnology-derived LAB products has received great attention in recent years,[8, 40, 41] and some food industry-derived residues have been used in these bioprocesses. The huge availability of these materials, along with their low cost or even cost-free, makes them alternative sources of substrates that can be used for bacteria cultivations.[42] Among common agro-residues that have been used in research using LAB, are whey and whey permeate,[8, 43, 44] fish viscera,[45] corn steep liquor,[40] and lignocellulosic materials in general.[17, 46, 47]

The use of bran for the production of lactic acid by *L. amylophilus* GV6 was investigated.[41] A significant production of lactic acid was obtained, suggesting that wheat bran is an interesting option for the production of this organic acid. A similar study was performed in which wheat bran and corn steep liquor were used as components of culture medium for production of *L. rhamnosus* biomass and lactic acid producing as high productivities as with traditional media components such as yeast extract.[40]

Another important industrial residue that has been used in cultures of LAB is cheese whey, which can be an ingredient of the fermentation medium for these microorganisms, with reports showing significant productions of biomass and high conversions of sugars into lactic acid, demonstrating that the cheese whey is a major source of nutrients to compose culture medium.[8, 44, 48] Starchy wastewater (by-product from the potato washing process) was used by Srisamai[49] for the production of probiotic *Streptomyces*, this residue contains high concentrations of starch, important ingredient for growth of probiotic. The authors reported that the biomass yields obtained were equivalent to those from conventional medium based on glucose.

Microencapsulation of potentially probiotic microorganisms

The microencapsulation of LAB has been researched in order to expand their use in several ways. Microencapsulated LAB were used in the production of lactic acid and fermented milk products. The immobilization techniques aim to increase the concentration of cells in the reactor, to increase productivity, to boost cell protection against toxic forms of oxygen and the often low freezing temperatures of applications. In food products, microencapsulation was investigated to protect cells against the bactericidal effects of gastric acid, to increase stability, and to maintain culture viability during storage.[50]

Several food products are receiving the supplementation of probiotic microorganisms because of the beneficial effects promoted by them.[51-53] However, the viability of these microorganisms is low,[12, 13, 20, 54, 55] showing reduced ability to survive and multiply in the host organism, thus loosing the benefits that the probiotic could otherwise promote.

In order to act as functional probiotics in foods, LAB should be metabolically stable and active in the product, keeping viability during the passage through the upper digestive tract in large quantities, and effectively adhering and colonizing the intestine. To overcome these barriers, microencapsulation techniques are being investigated as a way of protecting the probiotic microorganism added to food products, with several approaches being proposed in the literature, including the preparation of emulsions, extrusions, coacervations, spray-drying, spray-chilling, electrospinning/electrospraying, and impinging aerosol technique. Table 2 presents some of the most important works reporting on this subject.

Emulsion

Emulsion is achieved by dispersing probiotic cells in an oil or organic phase. The emulsion is usually broken by the addition of $CaCl_2$ and the microcapsules are collected by centrifugation.[56] The tolerance of *L. casei* 298 NCDC to low pH (1.5), high bile salt concentrations (1 % or 2 %), and heat processing (55 to 65 °C, for up to 20 min), encapsulated using this technique with different concentrations of sodium alginate (2, 3, or 4 %) was studied and results showed that the encapsulation improved cell resistance to the conditions tested when compared to the free cells, with viability increasing proportionally to the concentration of sodium alginate used.[22] The survival of probiotics in yogurt using cryoprotectants, prebiotics, and microencapsulation in alginate by emulsification was investigated.[57] Microencapsulation improved viability of probiotics in the product after six months of storage at 4 °C (8.67 log CFU g^{-1}, compared to 8.19 log CFU g^{-1} for the free-cells control), and 21 °C (6.79 log CFU g^{-1} and 5.63 log CFU g^{-1}, respectively).

The effects of microencapsulation by emulsion of several species of *L. plantarum* in alginate matrixes coated with whey proteins and submitted to simulated gastric fluid were studied, showing that entrapped coated cells had a final survival of 7.76 log CFU g^{-1}, 6.67 log CFU g^{-1}, and 5.81 log CFU g^{-1} for strains 299v, CIP A159, and 800, respectively, compared to only 2.19 log CFU g^{-1}, 1.89 log CFU g^{-1}, and 1.65 log CFU g^{-1}, for the respective controls of free suspended cells. The initial cell count was 10.04 log CFU g^{-1} for all strains. The control of uncoated alginate beads showed total loss of viability of cells after 60 and 180 min of exposure to simulated gastric medium.[21]

Mokarram[58] investigated the influence of coating of sodium alginate beads, prepared by the emulsification technique, with either one or two layers of sodium alginate on the survival of *L. acidophilus* PTCC1643, and *L. rhamnosus* PTCC1637. Cells were

sequentially exposed to simulated digestive solutions of gastric juice (pH 1.5, 1 h) and intestinal juice (pH 7.25, 2 h). Results showed that sodium alginate coatings protected cells to these simulated conditions: the number of surviving cells were 6.5 log CFU ml^{-1} for *L. acidophilus* and 7.6 log CFU ml^{-1} for *L. rhamnosus* when they were doubled-layered. A much lower cell counting of 2.3 and 2.0 log CFU ml^{-1}, for each strain respectively, were found in the control of free cells.[58]

Extrusion

In the extrusion technique, a solution of polymers, such as alginate, is first mixed with the microbial cells and then extruded through an orifice as droplets into the solution of a cross-linking agent, such as calcium chloride.[7, 59, 60]

In a comprehensive study of microencapsulation by extrusion, the viability of LAB exposed to simulated gastric fluid was accessed. *Lactobacillus* species (*L. casei subsp. rhamnosus* CCRC 12321, *L. acidophilus* CCRC 14079), and *Bifidobacterium* species (*B. longum* CCRC 14605, *B. bifidum* CCRC 11844) were microencapsulated by extrusion using combinations of varying concentrations of sodium alginate (1 to 3 %), peptides (0 to 1 %), fructooligossacharides (FOS, 0 to 3 %), and isomaltooligossacharides (IMO, 0 to 3 %). When exposed to simulated gastric fluid, the best combination of materials that improved viability was a composition of 3 % sodium alginate, 1 % peptide, 3 % FOS, and 0 % IMO. Using this combination of materials, *Lactobacillus* spp. showed 8.46 log CFU g^{-1} after 1 h of exposure to simulated gastric fluid (initial count of 8.68 log CFU g^{-1}), whereas *Bifidobacterium* spp. went from 7.86 log CFU g^{-1} to 7.72 log CFU g^{-1} under the same conditions.[23]

The microencapsulations by extrusion or by emulsification were compared by Özer[20] concerning the viability of *B. bifidum* Bb-12 and *L. acidophilus* LA-5 LAB strains that

were added to white-brined cheese. The authors reported that both techniques were effective in preserving the viability of cells during the maturation period compared to control (free cells). Both microencapsulation techniques were effective in keeping the numbers of probiotic bacteria above the required levels of the minimum therapeutic dosage (>10^7 log CFU g^{-1}) with viability losses of approximately 1 log, whereas the counts of free cells controls decreased by approximately 3 logs. No adverse effects were observed in the sensory evaluations of products that were added of microencapsulated probiotics.

Coacervation

The coacervation technique has been used to encapsulate enzymes, flavor oils, as well as microbial cells.[61-63] This microencapsulation technique utilizes phase separation of one or more incompatible polymers from the initial coating polymer solution under specific temperature, pH, or composition of the solution. The incompatible polymer is added to the coating polymer solution and the dispersion is stirred. Changes in the physical parameters lead to the separation of incompatible polymer and deposition of dense coacervate phase surrounding the core material resulting in the production of microspheres.[61, 62, 64]

Coacervation has shown to have a good encapsulation capacity and controlled liberation of the core material from the microspheres, showing resistance towards mechanical stress, temperature, and pH changes in media.[62] The pH resistance is especially useful for encapsulation of probiotics that are required to be released when exposed to the milieu pH in the large intestine.[63, 65] A technical development of polymeric microcapsules of whey protein isolate (WPI) and gum arabic (GA) (both, 3 % mass fraction solutions), mixed at 2:1 weight ratio, to microencapsulate *L. paracasei* subsp.

paracasei (E6) and *L. paraplantarum* (B1) was tested in acidic environments. The entrapment of these lactobacilli in the complex coacervate structure enhanced cell viability when exposed to a low pH environment (pH 2.0). Both encapsulated strains retained high viabilities in simulated gastric juice (above 73 % survival), compared to less than 19 % for free cells. Furthermore, after 60 days of storage at 4 ^{o}C, pH 4.0, the viability of microencapsulated cells were still high, (<86 %), implying improved protection in comparison with the free cells (< 59 %).[66]

Spray-drying

The spray-drying technique consists in the atomization of a suspension of viable LAB cells immerse in a polymeric solution into hot drying air, and fast evaporation of water.[60, 67, 68] The encapsulated product is separated as a dry powder from the conveying air in a cyclone. Various operational conditions, such as inlet air temperature, feed temperature, product feed rate, airflow, and outlet air temperature, need to be optimized in order to produce small and uniform microspheres.[60, 69, 70] The appropriate adjustment of the inlet air temperature is important because low air temperatures reduce the rate of water evaporation, resulting in microspheres with high density membranes and poor flow properties, whereas excessively high air temperatures can adversely affect cell viability. Moreover, feed temperature adjustment is crucial to modify the viscosity of the polymer solution and, in turn, its capacity to be sprayed in a homogeneous form.[59, 60] One common approach in spray-drying LAB and probiotic bacteria in general has been the use of spray-drying whey as cell carrier. Some experiments have shown that spray-dried LAB with probiotic characteristics (*B. breve* R070 (BB R070), *B. longum* R023 (BL R023), and *L. rhamnosus* GG) in denatured whey proteins and milk fat had increased viabilities when exposed to simulated gastric

fluid, compared to free cells.[12] Spray-dried *L. rhamnosus*, CRL 1505 using pectin and pectin–whey protein for the encapsulation resulted in increased cell survival (7 log CFU ml^{-1}), compared to free cells (2 log CFU ml^{-1}), when exposed to simulated gastric juice.[71] However, the authors reported that the incorporation of whey protein into the pectin matrix reduced the protective effect of the microcapsules, possibly caused by whey protein, which interferes with the gelation of pectin, producing a weaker gel with a bigger swelling capacity.[71]

An adaptation of the method of spraying cells to microencapsulated them is the use of spray-chilling, which consists of the dispersion of the core material into a warm coating material and the spraying through a heated nozzle into a controlled environment, where the encapsulant solidifies to form the microcapsule.[72]

L. acidophilus was co-encapsulated with a mixture of either inulin or polydextrose (prebiotics) in solid lipid microparticles (SLM), using the spray-chilling technique.[73] The best results in terms of long term storage was achieved using the formulation of *L. acidophilus* and polydextrose, combination that resulted in the maintenance of high cell viabilities for 120 days at -18, 7 and 22 °C. The SLM produced in this study demonstrated to be an interesting vehicle that could be applied in food formulations, given their specific morphology and insoluble nature.[73]

Spray chilling/cooling/congealing

This method of microencapsulation is similar to spray drying, producing small droplets, with the important difference that chilling involves the injection of cold air instead of hot air, which allows the solidification of the particle.[74] Spray chilling was produced to solve the problem of cell damage caused by exposure of microorganisms to high temperatures during spray drying.[60, 74, 75] Concerning probiotics, *L. acidophilus* (LAC-

04) was microencapsulated with cocoa butter using the spray-chilling technology. The results showed that microencapsulated cells of *L. acidophilus* were more resistant to simulated gastric and intestinal fluids than the free cells, with the viability of the encapsulated cells being 67 % higher than that of free cells. The microencapsulated cells showed reasonable shelf life after 90 days when stored at -18 °C, but authors claimed that storage survival should be improved for practical use.[75]

Freeze-drying

This is a new concept to microencapsulate microbial cells that can be applied to LAB and probiotics. In this method, cells are first frozen and then dried by sublimation of the solvent, normally water, under high vacuum.[76, 77] However, this method can cause damage to the cell membrane because of crystal formation and the stress condition of high osmolarity. Several cell-protectants have been used in freeze-drying to keep viability of microbial cells during dehydration, such as glucose, trehalose, maltodextrine, skim milk powder, and whey protein. Cryoprotectants may also be incorporated to media before fermentation to assist in the adaptation of microbial cells to the environment.[57, 78] These cryoprotectants accumulate within cells, decreasing the osmotic difference between the internal and external cell environments.[79, 80]

The cell cryoprotectans cellobiose, lactose, sucrose, and trehalose were tested in the freeze-drying of probiotic *Bifidobacterium infantis* UV16PR. The viability of cells was tested in formulations with milk, red-beet juice, and grape juice. The best results were 10 % of trehalose in milk formulations (8.2 log CFU ml^{-1} after 2 weeks storage); 5 % of cellobiose in formulations with red-beet juice (7.3 log CFU ml^{-1} after 4 weeks storage) and grape juice (7.3 log CFU ml^{-1} after 4 weeks storage), compared to free-cells

preparations, that showed survival rates of 7.5, 4.9, and 4.8 log CFU ml^{-1}, respectively.[78]

Electrospinning/electrospraying

A new technology for the microencapsulation of probiotics that is being investigated is the use of electrospinning/electrospraying, a method that enables the formation of capsules - or spheres - with smaller sizes than those obtained in the formation of gels, in the micro to nano scales. Electrospinning/electrospraying does not require high temperatures, being a simple technique capable of producing either fibers or capsules.[81-83] The technique of electrospraying is based on the principle of liquid atomization using electrical forces. The liquid flowing out of a capillary nozzle, at high electric potential, is forced by the electric field to be dispersed into fine droplets. The size of electrospray droplets can range from hundreds micrometers down to tens of nanometer.[84] The technique of electrospinning - a variation of electrospraying - is a process that has been used to fabricate fibers with submicron-scale diameters. A high voltage is applied to form an electrically charged jet of polymer solution or melt, which dries or solidifies to leave a polymeric fiber.[85] One electrode is placed into the spinning solution and another connected to a collector. Electric field is subjected to the end of a capillary tube containing the polymeric fluid held by its surface tension, inducing a charge on the surface of the liquid.[85]

Few works have dealt with the use of electrospinning/electrospraying to encapsulate microbial cells, especially for LAB and probiotics. In one of then, the electrospinning technique was employed to encapsulate *L. acidophilus* in fractions of soluble dietary fibers. A significant survival of the probiotics were achieved when stored for 21 days at

4 ºC, (78.6 to 90 %), and the thermal resistance of the encapsulated probiotics suggested a possible thermal protection of cells when delivered in heat-processed foods.[86]

In another attempt, the probiotic bacterium *B. animalis subsp. lactis* Bb12 was encapsulated using the electrospinning/electrospraying technique and concentrated whey and carbohydrate pullulan as matrixes. Results demonstrated that the concentrated whey produced a better protection concerning cell viability compared to pullulan, demonstrating ability to enhance cell survival during storage at 4 and 20 ºC and at various conditions of relative humidity (0, 11, 53 and 75 %).[83]

Impinging aerosol technology

Another recent technology, developed by Sohail,[87] employed for the continuous encapsulation of probiotics, is based on the separate impinging aerosols technology[88] of sodium alginate solution and calcium chloride cross-linking solution to produce water insoluble cross-linked alginate micro-beads (40 μm). According to the authors, solvent and heat are not used in the process, thus the method is suitable for encapsulating heat-labile and solvent-sensitive materials. The method can be easily scaled-up and microcapsules can be produced be spray or freeze-drying.[87] Probiotic alginate micro-beads of *L. rhamnosus* GG and *L. acidophilus* NCFM, were prepared using the novel impinging aerosols, in which the Ca-alginate micro-beads were coated, with a protective layer of chitosan.[89] Extruded macro-beads obtained by the conventional method were used to compare with the micro-beads prepared by the impinging aerosols technique. Results showed that the impinging aerosol micro-beads and those obtained using the conventional method offered similar protection to *L. rhamnosus* GG in the acid and bile tolerance study. However, when the authors used *L. acidophilus* NCFM, the extruded macro-beads were more effective than micro-beads in

protecting against acid and bile solution. For both bacteria probiotics, the coating of beads with chitosan improved survival. It was possible to confirm that the novel impinging aerosols methodology was as effective as other alginate gel encapsulation techniques to protect cells.[89] The same authors[87] studied the use of this technology in food models, with mixed results. Microencapsulated *L. rhamnosus* GG and *L. acidophilus* NCFM were tested for their survivability under acidification in orange juice at 25 °C for nine days or at 4 °C for thirty-five days of storage. Although free cells of *L. rhamnosus* GG had shown good survivability in orange juice at both temperatures, the same was not observed for free cells of *L. acidophilus* NCFM. The microencapsulation of these two probiotics did not significantly enhance the survivability in any of the tested conditions. One important observation that can also be draw from these experiments is that, besides the materials and techniques used in microencapsulation, general cell response to stress will be strain-dependent.

Although encapsulation and microencapsulation of LAB by various techniques have been proved successful, an important challenge remains to be addressed, which is the scaling up of microencapsulation process for commercial production. In special, there is still a need for the development of processes and equipment for large scale microencapsulations that could further improve commercialization of products formulated containing immobilized probiotics that are both economically viable and showing good acceptance by people.[90]

Table 2. Encapsulation of potentially probiotic microorganisms using different techniques of immobilization and materials.

Technique	Microrganism	Microencapsulation materials	References
	B. breve	Alginate	(119)
	L. casei	Carrageenan/locust bean gum	(120)
	L. rhamnosus, *B. longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. paracasei*, *B. lactis* type Bi-04 and*B. Lactis* type Bi-07	Alginate	(121)
Emulsion	*L. acidophilus* LA1	Alginate and starch	(122)
	L. paracaseis sp. *Paracasei* F19 and *B. lactis* Bb12	Sodium caseinate N 94	(123)
	L. plantarum BL011	Alginate, chitosan and pectin	(13)
	L. acidophilus	Alginate and starch	(124)
	L. casei	Alginate and pectin	(125)
	L. plantarum NCIMB 8826 *and B. longum* NCIMB 8809	Alginate, pectin, chitosan, gelatin and glucomannan	(126)
	L. acidophilus and *B. bifidum*	Alginate	(20)
	L. rhamnosus GG and *L. acidophilus* NCFM	Alginate and unmodified native corn starch	(127)
	L. rhamnosus	Whey protein	(128)
Extrusion	*L. acidophilus*	κ-carrageenan	(129)
	B. lactis	Gellan and xanthan gum	(130)
	L. reuteri	Alginate	(131)
	L. plantarum NCIMB 8826	Sodium alginate and chitosan	(132)
	L. plantarum 15HN	herbal-based biopolymers	(133)
	B. lactis (BI 01) and *L. acidophilus* (LAC4)	Casein and pectin	(62)
Coacervation	*L. paracasei* subsp. *Paracasei* (E6) and *L. paraplantarum* (B1)	whey protein isolate and gum Arabic	(66)
	L. acidophilus LA-5	Pectin and whey protein	(134)
	L. paracasei	Skim milk	(135)
	Bifidobacterium Bb-12	Skim Milk, Orafti ®Synergy1, inulin, and Oligofructose	(136)
	L. acidophilus and *B. longum*	Maltodextrin and gum Arabic	(137)
	L. acidophilus and *L. lactis* sp	Vege oil and sodium caseinate	(138)
	L. reuteri DSM 17938	Alginate and calcium chloride	(139)
	L. acidophilus NRRL B-4495 and *L. rhamnosus* NRRL B-442	Maltodextrine	(140)
	L. rhamnosus GG	Whey protein isolate, maltodextrine, inulin and glucose	(141)
	Bifidobacterium Bb-12	Whey protein	(142)
	L. kefir CIDCA 8321 and 8348	Skimmilk, sucrose, mono sodium glutamate and frutooligosaccharides	(143)
Spray-drying	*L.casei* UFPEDA	Maltodextrin	(144)
	L. rhamnosus B442	Native rice starch and inulin	
	L. salivarius NRRL B-30514	Whey protein isolate, sodium caseinate and pectin	(145)
	L. zeae LB1, *L. reuteri* S64 and K67	Sodium caseinate	(146)

	Saccharomyces cerevisiae var. boulardii	Gelatin, whey protein concentrate, modified starch, maltodextrin, pea protein isolate and gum Arabic	(147)
	L. acidophilus LA-5, *B. animalis* subsp. *lactis*BB-12and *Propionibacterium jensenii* 702	Goat's milk	(148)
Spray chilling/cooling/congealing	*L. acidophilus* (LAC-04)	Cocoa butter	(149)
	L. acidophilus (LAC-04)	Inulin and polydextrose	(73)
	L. plantarum (MTCC 5422)	Fructooligosaccharide, whey protein isolate and denatured whey protein isolate	(150)
Freeze drying			
	L. plantarum (mtcc 5422)	Whey protein	(151)
	L. salivarius subsp. s*alivarius* (UCC 500)	Sucrose, trehalose, skimmilk	(152)
	B. animalis subsp. *lactis* Bb12	Whey protein concentrate and Pullulan	(83)
Electrospinning/electrospraying			
	L. acidophilus FTDC 8933	Soybean solid waste, oil palm trunk and oil palm frond	(86)
Impinging aerosol technology	*L. rhamnosus* GG (ATCC 53103) and *L. acidophilus* NCFM	Alginate	(87), (89)

Conclusions and Perspectives

The growing interest of people in their personal health is pushing the development of functional foods, with strong emphases on foods that contain probiotic microorganisms and prebiotics. In particular, probiotics are associated with improving health of hosts by modifying or fortifying the functional microbial population in the gut. In terms of production of probiotics, the fed-batch fermentation, according to results reported by different authors, represents the best technology for achieving high cell productivities of probiotic microorganisms and their metabolites. Regarding the use of immobilized cells of probiotic microorganisms by different protocols and matrixes agents, this technology represents a promising alternative to increase microbial cell resistance to environmental stress. When added to food products, the immobilized cells remain viable during the shelf life of the products. One challenge remaining is the scaling-up of immobilization techniques.

Alternative media for the production of LAB and probiotic microorganisms using agro-wastes and other by-products have proved to be feasible in terms of biomass and lactic acid productions and could reduce costs of production, usually very high because of sophisticated media composition. It is possible to visualize, as future prospects, the need of production of these microorganisms in innovative cultivation media free of animal-derived products, in view of the increasing numbers of people with lactose intolerance, milk protein allergy, and high cholesterol health issues. Besides these health associated benefits, probiotic bacteria produced using only all-vegetal media will be extremely appealing to the increasing market of vegetarian and vegan consumers, who will benefit of products containing potentially probiotic microorganisms that are free of ingredients of animal origin.

Acknowledgments

This work was supported by grants from Brazilian Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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CAPÍTULO III – *LACTOBACILLUS PLANTARUM* BL011 CULTIVATIONS IN INDUSTRIAL ISOLATED SOYBEAN PROTEIN ACID RESIDUE

Este artigo foi submetido ao periódico ***Brazilian Journal of Microbiology***

Lactobacillus plantarum BL011 cultivations in industrial isolated soybean protein acid residue

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ABSTRACT

In this research, physiological aspects of *Lactobacillus plantarum* BL011 growing in a new, all-animal free medium, were evaluated in bioreactors aiming at the production of this important lactic acid bacterium. Cultivations were carried out in submerged batch bioreactors, using the Plackett-Burman (PB) methodology to evaluate the influence of temperature, aeration rate, stirring speed, the concentration of liquid acid protein residue of soybean (LAPRS), soy peptone, corn steep liquor, and raw yeast extract. Results showed that all variables, with the exception of corn steep liquor, significantly influenced biomass production. The best condition was applied to bioreactor cultures, producing a maximal biomass of 17.87 g L^{-1}, whereas lactic acid, the most important lactic acid bacteria metabolite, peaked at 37.59 g L^{-1}, corresponding to a productivity of 1.46 g L^{-1} h^{-1}. This is the first report on the use of LAPRS medium for *L. plantarum* growth. These results support the industrial use of this system as an alternative to

produce probiotics without animal-derived ingredients in order to obtain high biomass concentrations in batch bioreactors.

Keywords: *Lactobacillus plantarum* BL011; lactic acid; liquid acid protein residue of soybean; Plackett-Burman design; industrial production of biomass.

INTRODUCTION

In bioprocesses, various constituents can be used to match culture media compositions for lactic acid bacteria (LAB) production; however, a key issue is the cost involved. The use of agro-residues to obtain biotechnology-derived products has received great attention in recent years (Brinques *et al*., 2010; Li *et al*., 2010) and several byproducts and raw materials of the food industry have been used in bioprocesses. The huge availability of these materials, along with their low or even very low costs, makes them alternative sources of substrates that could be used for bacteria cultivations (Silva *et al*., 2009). Among common agro-residues are whey and whey permeate (Brinques *et al*., 2010), starchy wastewater (Srisamai *et al*., 2013) corn steep liquor (Li *et al*., 2010), and lignocellulosic materials in general (Wee and Ryu, 2009; Abdel-Rahmana *et al*., 2011). The production of soy protein isolate and soy protein concentrate, both in very large demand in the world market, generates liquid acid streams of protein-rich residue, as a result of the washing steps and separation of the isolate soy protein. This liquid fraction comprises of sugars and proteins of low molecular weight. This agro-residue is discharged as an industrial effluent and it has never been tested in bioprocesses before.

From the nutritional point of view, there is a growing interest of professionals and consumers in healthier diets that, in addition of providing basic nutrients, would

also play beneficial roles on health, with probiotics among the most important components of such diets (Soccol *et al*., 2010; Keservani *et al*., 2010; Bhat and Bhat, 2011). The current definition of probiotics, given by the World Health Organization, states that these are live microorganisms that, when administered in adequate amounts in diets, will confer health benefits for the host, human or animal. Bacteria of the genera *Lactobacillus* and *Bifidobacterium* are classified among the main probiotics considered safe for food and feed use, and produced in industrial scales (FAO/WHO, 2001). Therefore, there is a great interest in screening for new potentially probiotic strains of LABs, such as *L. plantarum.*

Traditionally, probiotics are generally added to - or are components of - dairy foods. However, there has been a growing demand for probiotics by nondairy consumers because of increasing vegetarianism as a dietary preference throughout the world, and a large part of world population possessing allergy to dairy products, caused either by lactose or milk proteins (Prado et al., 2008; Nualkaekul et al., 2011; Céspedes et al., 2013). These issues, associated with concerns of occurrence of bovine spongiform encephalopathy by a part of consumers, especially in Europe, have increased the demand for all animal-free foods (Horn *et al*., 2005) and there is a strong growing demand for new products to attend these markets. In the case of probiotics, it would be highly desirable if technology could deliver biosystems for cell growth free of any animal components in order to prevent the transfer of these constituents to the final product (Heenan *et al*., 2002).

Among probiotic bacteria, or potentially probiotic, depending on the strain, *L. plantarum* stands up as a versatile lactic acid bacterium, found in a variety of foods and other environments such as the human gastrointestinal-tract (Siezen and Van Hylckama Vlieger, 2011). *L. plantarum* is safe to be use in food products (Bernardeau *et al*.,

2008). This probiotic has been studied by several authors, using different agro-residue substrates for biomass production, as well as for its metabolites of interest, especially lactic acid. Such substrates are malt, wheat, and barley extracts (Charalampopoulos and Pandiella, 2010), stillage and sugar beet molasses (Krzywonos and Eberhard, 2011), coffee husk, tamarind seed powder, ground nut oil cake, wheat and rice brans, bengal gram powder, black and green gram flours, barley, millet, ragi, oats, corn and rice flours (Natarajan and Rajendran, 2012), york cabbage (Jaiswal *et al*., 2012), quinoa, and wheat slurries (Dallagnol *et al*., 2013).

In this context, the aims of this research were to investigate the growth and fermentation activity of *L. plantarum* BL011, a strain isolated from Serrano cheese (De Souza *et al*., 2003), when using a new, alternative culture medium, which is completely free of animal-derived components and cheap. Growth and nutritional conditions that could have an influence on biomass formation were investigated using the Plackett-Burman design methodology. We also followed and analyzed the production of lactic acid along the process, because this is the most important metabolite of LAB. Finally, the process was scaled-up to submerged cultures in batch bioreactors.

MATERIALS AND METHODS

Microorganism and chemicals

The strain of *L. plantarum* BL011, isolated by our group from Serrano cheese and described elsewhere (De Souza *et al*., 2003), was used in this study. This strain was identified as *L. plantarum* BL011 and it is kept at Microbiology Culture Collection of BiotecLab (UFRGS, Porto Alegre, Brazil). The strain was identified at DSMA Laboratory (Mogi das Cruzes, SP, Brazil), by comparing the 16S rRNA amplicon

sequences with GenBank databases (access number AB5989861), which showed 100 % homology with *Lactobacillus plantarum* genus and species. Working stocks of cultures were maintained in 20 % (volume fraction) glycerol suspension frozen at -22 °C ± 1 ºC.

Unless otherwise stated, all chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich, St. Louis, USA.

Inoculum preparation

Erlenmeyer flasks (2 000 mL) containing 400 mL MRS (De Man *et al*., 1960) were inoculated with 1.5 mL glycerol stock culture and incubated at 37 ºC ± 1 ºC in a rotatory shaker (MA 830, Marconi, Piracicaba, Brazil) at 180 rpm and grown to optical density (OD) 1.0 at 600 nm. The cells were harvested by centrifugation (Hitachi, Himac CR 21E, Tokio, Japan) at 3 500 *g* for 15 min at 4 ºC ± 1 ºC. The cell pellet was washed and resuspended directly into the cultivation broth (150 mL), the composition of which was varied accordingly to the experimental design described in Tables 1 and 2. This procedure was used as the standard inoculum preparation for all experiments.

Liquid acid protein residue of soybean (LAPRS) preparation, and concentration

The LAPRS is the liquid fraction resulting of the washing and separation steps during the isolate soy protein production. This liquid fraction comprises of sugars and proteins of low molecular weight. The LAPRS was kindly donated by the Dupont Company (Esteio, Rio Grande do Sul, Brazil). This residue was collected in the industrial plant in the precipitation stage, which is the first unit operation in the wastewater treatment plant. The liquid was immediately stored in 5 000 mL polypropylene containers, sealed and transported under refrigeration to the laboratory

where it was immediately vacuum-concentrated at 45 °C ± 1 °C for 4 h and subsequently stored at - 22 ºC ± 1 °C until the experiments. This operation was carried out in order to concentrate the sugar content of samples.

The LAPRS, as collected in the industry, had the following composition (in g L^{-1}): total sugars, 10; protein, 2; lipids, 1; ashes (mineral salts), 2; pH, 4.6. The analyses of protein, lipids, ashes, and total sugar were quantified according to the AOAC methods (AOAC, 1995).

Prior to be used in the cultivations, LAPRS was concentrated to 50 or 25 % of its original volume, having the final sugar concentrations of 20 g L^{-1} (LAPRS20, for the screening variables design), and 40 g L^{-1} (LAPRS40, used in the all other experiments), respectively.

Cultivation procedures

Screening of variables

A Plackett-Burman (PB) design was used in order to determine which nutrients and conditions could have a significant effect on *L. plantarum* BL011 biomass formation, the dependent variable, which is presented in Table 1 (Plackett and Burman, 1946). The concentrations and values for the independent variables were fixed based on the general values found on the literature for studies with this microorganism and on a previous research of our group (Brinques *et al*., 2010). Lactic acid production was also followed in these experiments because it is the most relevant metabolite of this bacterium (Table 2). Seven real and eight dummy variables were screened in 19 trials, with triplicates at the central point. The minimal and maximal ranges selected for the seven parameters are presented in Table 2, in which each column represents an

independent variable and each row represents a trial. Variables with confidence levels > 95 % were considered to have significant influence on biomass production.

In the investigation of variables with a significant effect on biomass production, cultivations were carried out with LAPRS20 medium, added of (in g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04. This mineral solution was also used, at the same concentrations, to all cultivations in this work. The other components of the medium were soy peptone, corn steep liquor, and raw yeast extract (autolyzed non-purified yeast extract; Prodesa, SP, Brazil), which is a cheap source of mineral and vitamins. A volume of 150 mL of inoculum was transferred to a bioreactor filled with 1 350 mL of medium, with compositions varying in accordance with the specific assay of the PB design.

Thse experiments were performed using a 2 000 mL Biostat B bioreactor (B. Braun Biotech International, Melsungen, Germany), with pH set at 5.5 ± 0.2 (Brinques *et al.*, 2010) and controlled by the automatic addition of 10 M NaOH or 1 M H_3PO_4. Dissolved oxygen concentration (DOC) of cultures was measured using a polarographic O_2 probe (Mettler-Toledo, Germany). The volumetric oxygen mass transfer rate (k_La) was calculated using the dynamic gassing out method (Sinclair and Cantero, 1990). In two experiments, the DOC was kept at a minimum of 30 % of saturation by controlling the agitation speed through a control-loop. Temperature, aeration rate, and stirring speed were set up according to the PB design (Table 2). A confirmation experiment using the best conditions found in the PB design was carried out in the same bioreactor described above, except for LAPRS concentration, which was doubled. The medium for this experiment was composed of 15 g L^{-1} of raw yeast extract and 1 500 mL of LAPRS40, and conditions were 25 °C, pH 5.5 ± 0.2, agitation 200 rpm, and aeration 4.5 vvm.

Table 1: Independent variables studied in the Plackett-Burman design for the cultivation of *L. plantarum* BL011.

Variables/level	Temperature (ºC)	Corn steep liquor ($g\ L^{-1}$)	LAPRS ($g\ L^{-1}$)	Peptone of soy ($g\ L^{-1}$)	Yeast extract ($g\ L^{-1}$)	Stirring speed (rpm)	Aeration rate (vvm)
-1	25	5	5	2	2	200	2.5
0	34	12.5	12.5	8.5	8.5	300	3.5
+1	37	20	20	15	15	400	4.5

Saccharification and co-saccharification in the bioreactor

In order to hydrolyze the polymeric sugars present in LAPRS, saccharification reactions of this substrate were carried out using commercial invertase through two different approaches: 1) saccharification followed by fermentation as independent and sequential reactions (separate hydrolysis followed by fermentation), and 2) co-saccharification and fermentation carried out as a single reaction (simultaneous hydrolysis and fermentation in the same vessel).

LAPRS hydrolysis was carried out using invertase from *S. cerevisiae* (Maxinvert L 10000, batch 409200451, DSM Food Specialties, The Netherlands. Enzyme activity (1 U) was defined as 1 µmol of glucose released from sucrose per minute at 50 °C ± 1 °C and 42 % sucrose (mass fraction)). For condition 1), a volume of 1 500 mL LAPRS40 was added of 18.21 U in a 2 L flask and incubated at 50 ^{o}C, pH 4.6, for 3 h, under agitation. The hydrolyzed solution was then added of the other nutrients and used for fermentation without further modifications. For condition 2), the co-saccharification and fermentation, the enzyme was directly added to the final medium, in the bioreactor, in the presence of cells, under the conditions of the fermentation. All trials were made in duplicates.

All cultivations were carried out in the bioreactors as described above, under the same conditions (25 °C, pH 5.5 ± 0.2, agitation 200 rpm, and aeration 4.5 vvm).

Analytical methods

Samples of 10 mL of culture broth were centrifuged at 3 500 *g* for 15 min at 4 ºC ± 1 °C. The cell-free supernatant was used for the quantification of sugars and lactic acid. Biomass was measured gravimetrically as dry weight of cells. Samples were centrifuged, washed twice with cold distilled water, and dried in pre-weighed plastic

tubes at 80 ºC ± 1 °C to constant weight in vacuum ovens. Total sugars concentration was determined by Dubois method using glucose as standard (Dubois *et al*., 1956). Lactic and acetic acids concentration were determined by HPLC (Shimadzu, Kyoto, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 × 7.8 mm) using 5 mM sulfuric acid as eluent at 65 °C ± 1 °C, flow rate of 0.8 mL min^{-1} and sample volumes of 20 μL. Samples were filtered through cellulose acetate 0.22 μm membranes prior to HPLC injection.

Data analysis

All experimental designs and results analyses were carried out using Statistica 10.0 (Statsoft, Tulsa, USA).

RESULTS AND DISCUSSION

Experimental design

Plackett–Burman design was used to evaluate the effects of concentrations of LAPRS, soy peptone, corn steep liquor, and raw yeast extract as medium components; and temperature, stirring speed, and aeration rate as cultivation conditions on *L. plantarum* BL011 biomass formation (dependent variable), and lactic acid production (important metabolite). Table 2 presents the PB experimental design for 19 trials with two study levels for each variable and the corresponding biomass production in 48 h of bioreactor cultivation, whereas in Table 3 is presented the statistical analysis of the studied variables. Except for the concentration of corn steep liquor, all other variables were found to be significant at the 95 % level for biomass production. The highest values of biomass formation (7.74 g L^{-1}) and lactic acid production (21.09 g L^{-1}), were obtained in test 5. The value of k_La, measured under these conditions, was 30 h^{-1} (200

rpm, 4.5 vvm, mid-exponential growth phase), indicating that this aeration condition could be adopted for scaling-up the process, independently allowing the test of other variables.

Temperature and steering speed had significant negative effects. Therefore, for the next set of experiments in the bioreactor, these variables were set at 25 ºC ± 1 °C and 200 rpm, respectively, the lower levels of them. Aeration rate showed significant positive effects, being fixed at 4.5 vvm. Corn steep liquor, the only variable that did not have a significant effect, was excluded for the next experiments. Finally, although statistically significant, peptone of soy showed a negative effect, and because it has a high cost, it was taken out of medium composition for the next experimental step.

Based on the results obtained in the PB design, experiments were carried out to confirm the best conditions for *L. plantarum* BL011 cultivation, which were fixed at: k_La of 30 h^{-1} (obtained using the combination of 200 rpm and 4.5 vvm), 25 ^oC, pH controlled at 5.5, raw yeast extract 15 g L^{-1}, and 1 500 mL of LAPRS40. The doubling of the total amount sugars of LAPRS, form 20 to 40 g L^{-1}, was proposed because this variable produced a positive and significant effect on the PB. Figure 1 depicts the kinetics of these cultivations. Maximal biomass obtained was 10.85 ± 0.03 g L^{-1}, values 28 % higher than the best condition found in the PB design, because of the higher sugar concentration. The highest lactic acid concentration was achieved at 12 h of cultivation, reaching 19.56 ± 0.73 g L^{-1}, with a productivity of 1.54 g L^{-1} h^{-1}, similar to values of the PB design. Acetic acid obtained was 18.43 ± 0.25 g L^{-1} in 48 h of cultivation. The remaining sugar left in cultivation might be a consequence of *L. plantarum* BL011 inability to completely hydrolyze all sugars present in the LAPRS, since this vegetable source contains some oligosaccharides, such as raffinose and stachyose, which require enzymatic breakdown.

Table 2: Plackett–Burman experimental design matrix for biomass and lactic acid production of *L. plantarum* BL011.

Trial no.	Variables[a]/level[b]															Biomass ($g\ L^{-1}$)	Lactic acid ($g\ L^{-1}$)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8		
1	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	4.39	7.06
2	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	0.44	4.74
3	-	+	-	-	-	+	+	-	-	+	+	+	-	+	-	2.21	9.46
4	+	+	-	-	+	-	-	+	-	+	-	-	+	+	+	2.31	13.63
5	-	-	+	-	+	-	+	-	+	-	+	-	+	+	-	7.74	21.09
6	+	-	+	-	-	+	-	-	+	-	-	+	-	+	+	1.46	12.06
7	-	+	+	-	-	-	+	+	-	-	-	+	+	-	+	3.07	15.88
8	+	+	+	-	+	+	-	+	-	-	+	-	-	-	-	4.61	20.77
9	-	-	-	+	+	+	-	+	-	-	-	+	+	+	-	0.90	6.29
10	+	-	-	+	-	-	+	+	-	-	+	-	-	+	+	0.58	5.69
11	-	+	-	+	-	+	-	-	+	-	+	-	+	-	+	0.70	8.10
12	+	+	-	+	+	-	+	-	+	-	-	+	-	-	-	2.16	14.62
13	-	-	+	+	+	-	-	-	-	+	+	+	-	-	+	4.39	10.01
14	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-	1.99	11.50
15	-	+	+	+	-	-	-	+	+	+	-	-	-	+	-	5.36	16.21
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	2.56	19.61
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.12	12.38
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.14	13.66
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.33	17.00

[a] X_1 temperature, at highest level of 37 ºC, central level of 31 ºC, and lowest level of 25 ºC; X_2 corn steep liquor concentration at highest level of 20.0 g L^{-1}, central level of 12.5 g L^{-1}, and lowest level of 5.0 g L^{-1}; X_3 Total sugars (LAPRS) at highest of 20.0 g L^{-1}, central concentration of 12,5 g L^{-1}, and lowest concentration of 5.0 g L^{-1}; X_4 peptone of soy at highest concentration of 15.0 g L^{-1}, central concentration of 8.5 g L^{-1}, and lowest concentration of 2.0 g L^{-1}; X_5 yeast extract at highest concentration of 15.0 g L^{-1}, central concentration of 8.5 g L^{-1}, and lowest concentration of 2.0 g L^{-1}; X_6 stirred agitation at highest level of 400 rpm, central level of 300 rpm, and lowest level of 200 rpm; X_7 aeration rate at highest level of 4.5 vvm, central level of 3.5 vvm, and lowest level of 2.5 vvm; D1, D2, D3, D4, D5, D6, D7 and D8 are dummy variables.

[b] (+) Highest concentration of variable; (-) lower concentration of variable; (0) central level of variable. Values of biomass and lactic acid were measured at 48 h of cultivation.

Table 3: Estimated effects for biomass production calculated from the results of the Plackett-Burman design.

Variables	Parameters	Effect	*P*-value
X_1	Temperature*	-1.58	0.0013
X_2	Corn steep liquor	0.14	0.1431
X_3	LAPRS*	2.19	0.0007
X_4	Peptone of soy*	-0.95	0.0037
X_5	Yeast extract*	1.66	0.0012
X_6	Stirring speed*	-0.90	0.0041
X_7	Aeration rate*	0.57	0.0103

Standard error = 0.58; *P*-values ≤0.05; R^2: 0.98

*Statistically significant at 95 % confidence level

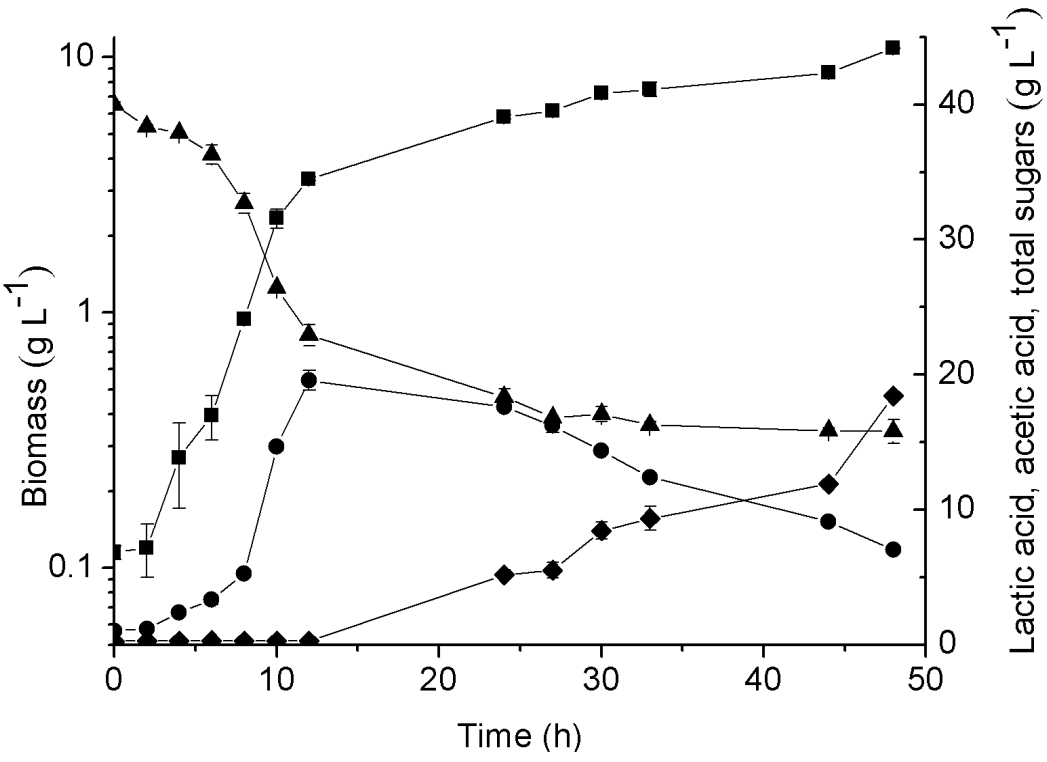

Figure 1. Time course of batch cultivations of *L. plantarum* BL011 in medium containing (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04; LAPRS, 40 (total sugars); yeast extract, 15. Culture conditions: 25 ºC ± 1; 4.5 vvm; 200 rpm, pH 5.5 ± 0.2; (■) dry cell weight; (●) lactic acid concentration; (♦) acetic acid concentration; (▲) total sugars concentration. Results are the mean of duplicates.

The main enzymes involved in the hydrolysis of these sugars are alpha-galactosidase and beta-fructosidase (invertase), which, in this work, showed to be of negligible activities for this strain growing under the conditions of the PB (results not shown).

Saccharification and co-saccharification in the bioreactor

Since it was confirmed the absence of invertase activity for *L. plantarum* BL011, two sets of enzyme-mediated saccharification experiments were devised intending to increase the hydrolysis of sugars of LAPRS, consequently offering more sugars for bacterial metabolism. The hydrolysis of LAPRS was done using invertase because this enzyme cleaves the β-1.2 bonds in raffinose and stachyose, producing melibiose and fructose, respectively. In the first experiment, saccharification was performed independently, with enzymatic hydrolysis followed by fermentation, and results of the cultivation kinetics are presented in figure 2. In the second experiment, saccharification and fermentation were carried out simultaneously, with results of the cultivation kinetics presented in figure 3. Although the growth kinetics were similar, both showing faster consumption of sugars when compared to the PB design, the hydrolysis of LAPRS before cultivation produced better results than co-saccharification and fermentation, perhaps because conditions for hydrolysis and fermentation were ideal, in contrast with the combined process. Obtained biomasses were 13.39 ± 0.10 g L^{-1} and 11.4 ± 0.48 g L^{-1}; lactic acid peaked at concentrations of 42.04 ± 0.08 g L^{-1} (24 h) and 37.36 ± 0.81g L^{-1} (24 h); and acetic acid peaked at concentrations of 32.35 ± 0.79 g L^{-1} (72 h) and 22.71 ± 0.19 g L^{-1} (72 h), respectively.

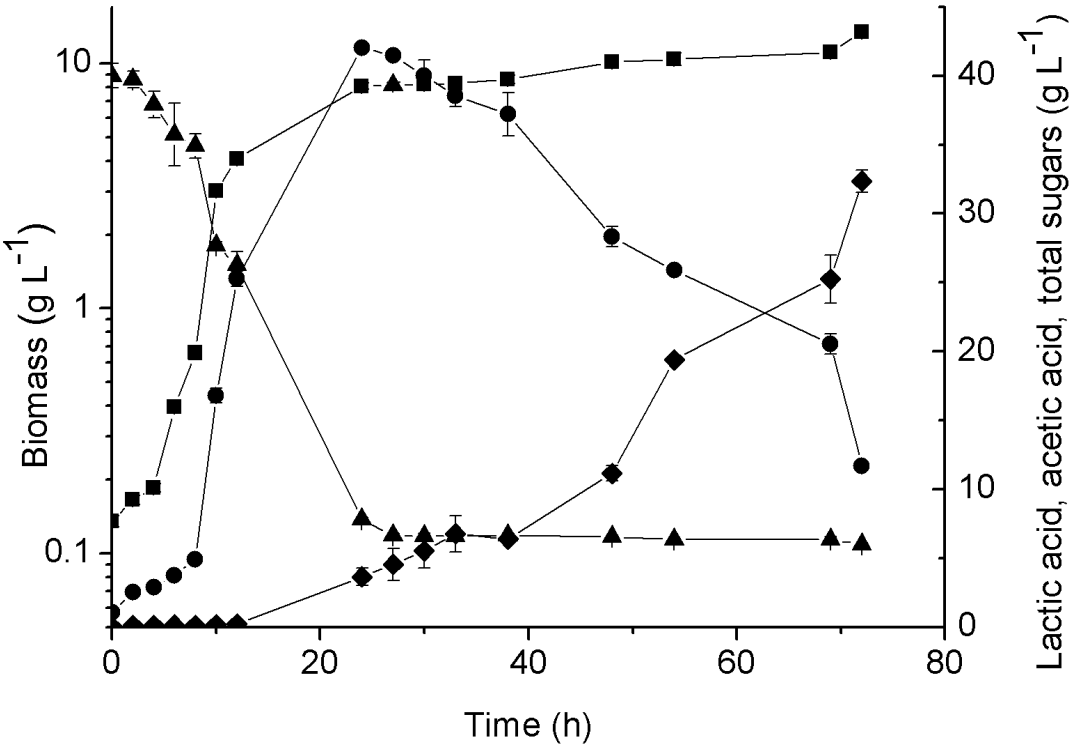

Figure 2. Time course of batch cultivation of *L. plantarum* BL011 in hydrolyzed LAPRS using invertase. Medium composition (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04; LAPRS, 40 (total sugars); yeast extract, 15. Culture conditions: 25 ºC ± 1; 4.5 vvm; 200 rpm, pH 5.5 ± 0.2; (■) dry cell weight; (●) lactic acid concentration; (♦) acetic acid concentration; (▲) total sugars concentration. Results are the mean of duplicates.

The productivities of lactic acid, measured at 24 h of cultivation were 1.71 g L^{-1} h^{-1} and 1.46 g L^{-1} h^{-1} for the saccharification and co-saccharification, respectively. Comparatively, Sikder *et al*., (2014) reported a lactic acid productivity of 1.24 g L^{-1} h^{-1} by *L. plantarum* NCIM 2912 growing in sugarcane juice medium containing 140 g L^{-1} (sucrose, 126; glucose, 8; and fructose, 6). Laopaiboon *et al*., (2010) obtained a lactic acid productivity of only 0.36 g L^{-1} h^{-1} in cultures of *L. lactis* IO-1 (JCM 7638), using hydrolyzed sugarcane bagasse as a substrate (30 g L^{-1}).

Results obtained using LAPRS40 for the growth of *L. plantarum* BL011, allowed for one of the highest biomass production compared to the literature. For

instance, Brinques *et al*., (2010) reported a maximal biomass of 10.2 g L^{-1} of *L. plantarum* using cheese whey (equivalent to 140 g L^{-1} of lactose) as medium carbon source. Gao *et al*., (2007) reported a final biomass production of 6.6 g L^{-1} of *L. rhamnosus* NBRC 3863, when growing this LAB in hydrolyzed fish waste. Mu *et al*., (2009) obtained a maximal cell density of 3.34 g L^{-1} of *Lactobacillus* sp. SK007 in a medium made up of glucose (30 g L^{-1}), yeast extract (30 g L^{-1}), and corn step liquor (47 g L^{-1}). Hwang *et al*., (2015) formulated a complex medium composed of different sugars and nitrogen sources for biomass production of *L. acidophilus* DGK. The authors reported maximal biomass production of 4.54 g L^{-1} under aerobic condition. Finally, *S. boulardii* MYA-769 was produced in a medium made up of grass juice feedstock extracted from ryegrass *Lolium perenne*, reaching a biomass of 4.96 g L^{-1} (Hull *et al*., 2014).

The results obtained using the LAPRS as the sole carbon source, demonstrated that this industrial residue is a suitable substrate for the growth of *L. plantarum* BL011, which has not been reported in the literature. An industrial plant of soy protein isolate can produce a large volume of acid residue, such as the company that supplied the material for this study, which has a monthly discharge of approximately 5×10^4 m^3 of LAPRS (data provided by the company). When compared against other residues reported in the literature, or even with synthetic media, LAPRS allows for a significant improvement of biomass formation. Moreover, the other component of the culture medium, the non-purified raw yeast extract obtained as the waste of beer production, has a very low cost compared against the traditional, purified yeast extract used for cell growth, further supporting the hypothesis that the culture medium used in this work is cheap and suitable for *Lactobacillus* cultivations.

Figures 2 and 3 shows the profile of lactic acid formation and its subsequent conversion into acetic acid by *L. plantarum* BL011, the most important metabolite of lactic acid bacteria. Partially, lactic acid supported a small diauxic growth towards the end of cultivation, but it was mainly converted into acetic acid. *L. plantarum* showed flexible and adaptive behavior for the first time in the chromosome of strain WCFS1, which encodes a variety of proteins related to sugar uptake and utilization (Kleerebezem *et al*., 2003), which allows *L. plantarum* to grow on several carbon sources. The genes encoding transporters are generally in clustered gene cassettes encoding enzymes and regulatory proteins implicated in sugar metabolism (Siezen and Van Hylckama Vlieger, 2011).

In conversion of lactic acid into acetic acid under aerobic conditions, one ATP is generated via lactate dehydrogenase, pyruvate oxidase, pyruvate dehydrogenase, pyruvate formate lyase, phosphotransacetylase, and acetate kinase (Quatravaux *et al*., 2006). This pathway also produces hydrogen peroxide and carbon dioxide. Hydrogen peroxide is generated by the conversion of oxygen through the manganese-dependent process. The final accumulation of acetic acid, instead of lactic acid, causes cell pH homeostasis (Quatravaux *et al*., 2006).

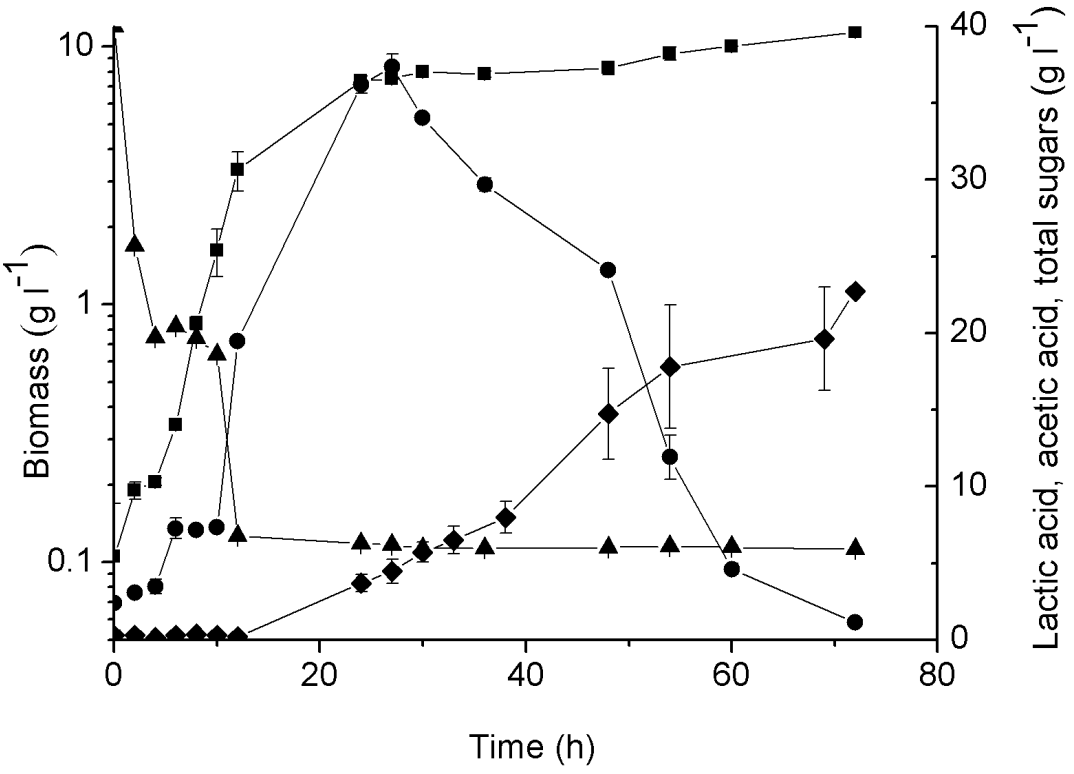

Figure 3. Time course of simultaneous saccharification and cultivation of *L. plantarum* BL011. Medium containing (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04; LAPRS, 40 (total sugars); yeast extract, 15. Culture conditions: 25 ºC ± 1; 4.5 vvm; 200 rpm, pH 5.5 ± 0.2 (■) dry cell weight; (●) lactic acid concentration; (♦) acetic acid concentration; (▲) total sugars concentration. Results are the mean of duplicates.

Experiments with DOC at minimum of 30 % saturation

Although aeration and agitation speed of turbines in the bioreactor were applied in order to guarantee adequate oxygen supply to cultures, the DOC could eventually fall below the critical oxygen concentration, which would cause a shift from aerobiosis to anaerobiosis. Therefore, we decided to run cultures under the best conditions, except for aeration, that was kept always above 30 % of DOC using a cascade loop in the aeration speed.

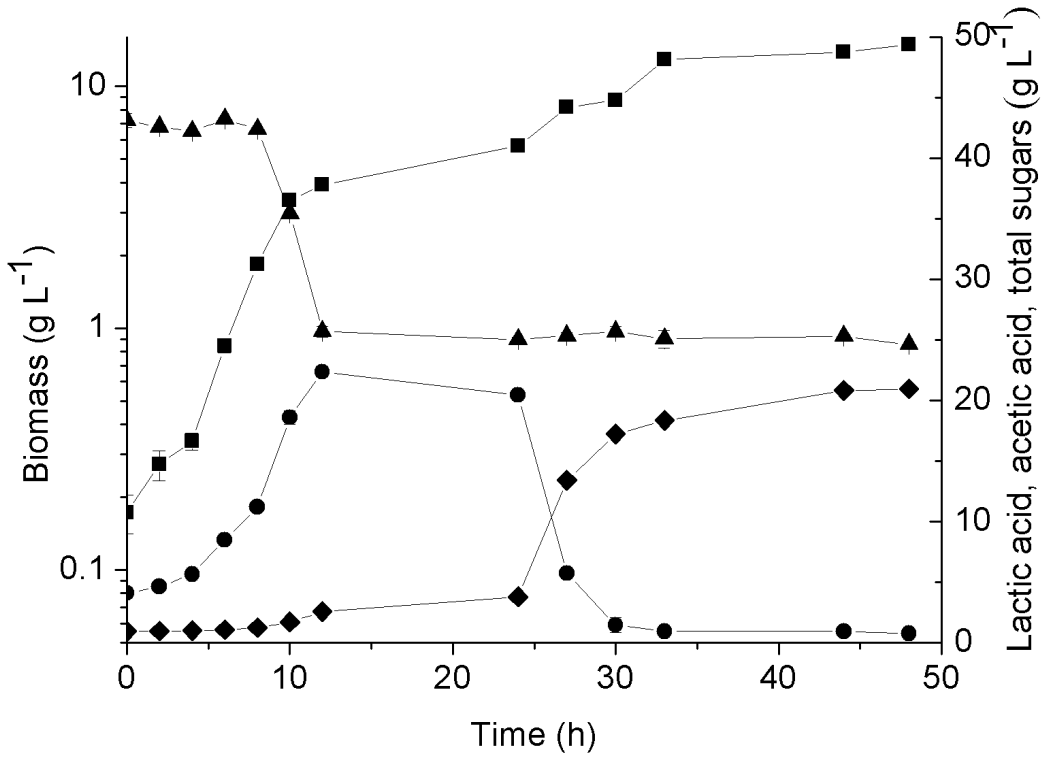

Figure 4. Time course of batch cultivation of *L. plantarum* BL011 under DOC of 30 % saturation or higher. Medium composition (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04; LAPRS, 40 (total sugars); yeast extract, 15. Culture conditions: 25 ºC ± 1; 4.5 vvm; 200 rpm, pH 5.5 ± 0.2 (■) dry cell weight; (●) lactic acid concentration; (♦) acetic acid concentration; (▲) total sugars concentration. Results are the mean of duplicates.

Results of these experiments, for the best conditions obtained for the PB design (Figure 1) and for the saccharification (Figure 2), are shown in Figures 4 and 5, respectively. Figure 4 shows a maximum biomass production of 14.86 ± 0.19 g L^{-1}, approximately 37 % higher than in conditions without DOC control. Lactic acid also increased, up to 22.34 ± 0.3 g L^{-1} (compared to 19.56 g L^{-1}). In the experiments where the LAPRS was hydrolyzed (saccharification), Figure 5, biomass was 17.87 ± 0.25 g L^{-1}, an increase of approximately 33.5 % relatively to the condition without DOC control, whereas lactic acid peaked at 37.59 ± 0.4 g L^{-1}. Under the condition of DOC controlled to 30 % of saturation, lactic acid was completely consumed to form biomass and acetic

acid, the two products at the end of cultivations. The result for biomass in the saccharification experiment, Figure 5, is the highest so far reported in the literature.

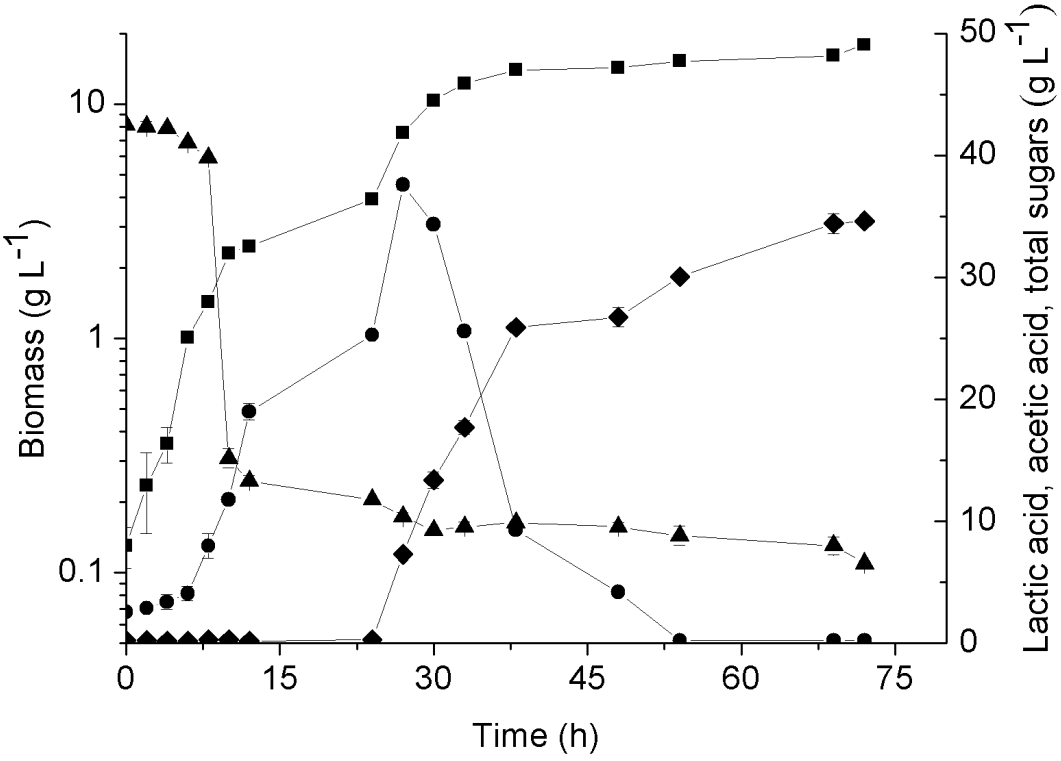

Figure 5. Time course of batch cultivation of *L. plantarum* BL011 in hydrolyzed LAPRS under DOC of 30 % saturation or higher. Medium composition (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04; LAPRS, 40 (total sugars); yeast extract, 15. Culture conditions: 25 ºC ± 1; 4.5 vvm; 200 rpm, pH 5.5 ± 0.2; (■) dry cell weight; (●) lactic acid concentration; (♦) acetic acid concentration; (▲) total sugars concentration. Results are the mean of duplicates.

CONCLUSIONS

The liquid acid protein residue of soybean (LAPRS), a vegetable carbon substrate, was successfully used to produce biomass of *L. plantarum* BL011 as a source of a potentially probiotic culture for food applications. Lactic acid was also produced in

large amounts, which could be explored, should its production be of interest. Production of biomass and lactic acid were high when compared to other reports on the literature. Plackett–Burman design provided an efficient method for the screening of the important cultivation variables of aeration and agitation speed, DOC, and the oxygen volumetric mass transfer, allowing for future scaling-up experiments. An innovative culture medium entirely made up without lactose or any animal-derived sources for the production of microorganisms could help the development of new industrial processes where lactose-free and animal-free ingredients are required. Further research is granted to test different bacteria, and perhaps yeasts, in this substrate.

Acknowledgements

The authors wish to thank CNPq and CAPES (Brazil) for the financial support and scholarships of this work.

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CAPÍTULO IV – ELECTROSPRAYING MICROENCAPSULATION OF *LACTOBACILLUS PLANTARUM* ENHANCES CELL VIABILITY UNDER REFRIGERATION STORAGE AND SIMULATED GASTRIC AND INTESTINAL FLUIDS

Este artigo foi submetido ao periódico ***Journal of Functional Foods***

Electrospraying microencapsulation of *Lactobacillus plantarum* enhances cell viability under refrigeration storage and simulated gastric and intestinal fluids

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ABSTRACT

Electrospraying was used to microencapsulate *Lactobacillus plantarum* in sodium alginate or in sodium alginate-citric pectin matrixes. Microencapsulated cells and control (free cells) were tested for survival when exposed to simulated gastric acid and intestinal juices (SGJ and SIJ). Results showed control viability losses of 6 and 4.2 log CFU mL^{-1} when exposed for 120 min to SGJ and SIJ, whereas microencapsulated cells decreased 2.9 log CFU mL^{-1} in SGJ, and 2.7 log CFU mL^{-1} in SIJ, showing significant resistance towards both juices. Refrigeration storage for 21 days showed cell survivals of 9.3 log CFU mL^{-1} (ALG) and 8.6 log CFU mL^{-1} (ALG-PEC) for microencapsulated cells, and 1.2 log CFU mL^{-1} for the control. FTIR spectra confirmed Ca^{2+} complexation of alginate and pectin structures obtained by electrospraying. Results demonstrate the

efficiency of this technique to increase the survival of *L. plantarum* in the digestive system of hosts.

Keywords: *Lactobacillus plantarum;* probiotics; electrospray; microencapsulation; cell viability.

1. INTRODUCTION

Probiotics are dietary supplements of live microorganisms presenting positive health effects for both humans and animals, while improving the intestinal microbial balance (Amine et al., 2014; Bron, Baarlen, & Kleerebezem, 2012; Vasiljevic & Shah, 2008; Vrese & Schrezenmeir, 2008). *Lactobacillus plantarum* is a lactic acid bacterium (LAB) found in a variety of environments (De Vries, Vaughan, Kleerebezem, & Vos, 2006). This microorganism is considered safe to be used in food products, being recognized and widely used as a probiotic (Bernardeau, Vernoux, Henri-Dubernet, & Guéguen, 2008; Kiessling, Schneider, & Jahreis, 2002; Naruszewicz, Johansson, Zapolska-Downar, & Bukowska, 2002; Nualkaekul & Charalampopoulos, 2011; Oxman, Shapira, Klein, Avazov, & Rabinowitz, 2001; Pochard et al., 2005).

When probiotics are added to food products, they may lose their viability (Amine et al., 2014; Brinques & Ayub, 2011), thus several techniques for the microencapsulation of LAB cells have been attempted as a form of cell protection (Antunes et al., 2013; Chen, Cao, Ferguson, Shu, & Garg, 2012; Fukui et al., 2010; Fung, Yuen, & Liong, 2010; Siaterlis, Deepika, & Charalampopoulos, 2009). Among materials used for microencapsulation of LAB, the most commonly researched are pectin (Brinques & Ayub, 2011; Nualkaekul, Cook, Khutoryanskiy, & Charalampopoulos, 2013, Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-

Ramírez, & Vernon-Carter, 2010), chitosan (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011; Lin et al., 2008; Liouni, Drichoutis, & Nerantzis, 2008), starch (Anal & Singh, 2007; Chan et al., 2011; Sabikhi, Babu, Thompkinson, & Kapila 2010), and natural gums such as sodium alginate (Amine et al., 2014; López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012; Martin-Dejardin et al., 2013).

Alginate, an anionic polymer extracted from brown seaweed, has been broadly used for the immobilization of probiotics because of its non-toxic nature, bioavailability, biocompatibility, low cost, and easy preparation as ionotropic gelation beads (Mokarram, Mortazavi, HabibiNajafi, & Shahidi 2009). Pectin, another natural polymer extracted from plant cell walls, is a linear chain of galacturonic acid units, linked by α-1,4 ligations, which are partially methyl esterified. Pectin has been widely used in foods and pharmaceutical products because of its excellent gelling capacity (Pérez, Mazeau, & Hervédu Penhoat, 2000). The use of these hydrocolloids for microbial immobilization entraps the cells within an array of fibres, whereas other techniques such as spray drying, freeze drying, coacervation, and cross-linking, may allow the leakage of free cells into the product, exposing them to the environment (Birnbaum, Kosmala, Henthorn, & Brannon-Peppas, 2000; Bruschi, Cardoso, Lucchesi, & Gremiao, 2003; López-Rubio et al., 2012; Mauguet et al., 2002).

Some studies reported the development of microencapsulation as an alternative for the encapsulation of probiotics, protecting cells against environmental stress such as extreme pH, temperature, excessive salinity, and enzyme degradation, thus increasing the cell viability (Alli, 2013; Brinques & Ayub, 2011; Nualkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos, 2012).

New technologies for microencapsulation of bacteria are being developed, among them, the technique of electrospray, which enables the production of small

capsules varying from the micro to the sub-micro sizes (López-Rubio, Sanchez, Sanz, Lagaron, 2009; López-Rubio et al., 2012; Torres-Giner, Martinez-Abad, Ocio, & Lagaron, 2010). It has been suggested that this technique could protect cell viability of immobilized LAB to be used as probiotics (Fukui et al., 2010; López-Rubio et al., 2012). However, research on this topic is still scarce and more work is needed to measure the stability of the immobilized system when incubated into simulated gastric and intestinal juices, and to better understand the behaviour of the microcapsules obtained by electrospray when this material is added to food products.

Considering these aspects, the aim of this work was to investigate the possibility of microencapsulating *L. plantarum*, a probiotic LAB, using the technology of electrospraying with sodium alginate and a mixture of sodium alginate-citric pectin as supports for the encapsulation. Following the development of microcapsules, we evaluated the viability of the encapsulated cells under cold storage and when incubated into simulated gastric and intestinal juices. Free cells of *L. plantarum* were compared to the microencapsulated cells as controls.

2. MATERIALS AND METHODS

2.1 Materials

Food grade sodium alginate (ALG) was purchased from Gastronomylab (Brasília, Brazil); low-methoxyl citric pectin was kindly provided by Plury Química (Diadema, Brazil); other reagents used in this research were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA).

2.2 Microorganism

A strain of *L. Plantarum* BL011, isolated by our group from Serrano cheese and

described elsewhere (De Souza, Dalla Rosa, & Ayub, 2003) was used in this study. This strain was identified as *L. plantarum* BL011 and it is kept at Microbiology Culture Collection of BiotecLab (UFRGS, Porto Alegre, Brazil). Working stocks of cultures were maintained in 20 % (volume fraction) glycerol suspension frozen at -22 °C ± 1 ºC.

2.3 Preparation of alginate-based microcapsules by electrospraying

Two types of gelling solutions were prepared and used in this research: a) 3 % (mass fraction) of sodium alginate (ALG, Solution 1); and b) 4 % of sodium alginate added of citric pectin (4 % mass fraction) (ALG-PEC, Solution 2). Solutions 1 and 2, added or not of bacteria cells, were introduced separately in a 1 mL plastic syringe and were electrosprayed under steady flow-rate of 2 mL h^{-1} using a stainless-steel needle of 0.06 mm of diameter. The voltage applied was 24 kV, the distance of the needle tip-to-collector was 15 cm. This procedure was carried out in an in-house apparatus. The formed microspheres were collected into a beaker containing a solution of calcium chloride (CaCl, 0.5M).

2.4 Characterization of ALG and ALG-PEC solutions

The pH and the conductivity of the solutions were measured using a pHmeter Digimed DMPH-2 and in a Digimed DM-31 (Digimed, Brazil), respectively, immediately after their preparations. The solutions viscosities were obtained using a rotational viscometer DV-II + PRO (*Brookfield*, England), using spindles SC4-18. All measurements were carried out at 25 ºC, in triplicates.

2.5 Characterization of microcapsules

2.5.1 Infrared Spectroscopy (FTIR)

The chemical characteristics of the electrosprayed microcapsules were assessed using an infrared spectrometer (FTIR, Perkin-Elmer 16 PC spectrometer, Boston, USA), in Attenuated Total Reflectance mode (ATR) between 4000-600 cm^{-1}, using 64 scans at a resolution of 4 cm^{-1}.

2.5.2 Scanning Electron Microscopy (SEM)

The morphologies of the microcapsules were obtained using a Scanning Electronic Microscopy (SEM) model JSM 6060, operating at an accelerated voltage of 10 keV, under high vacuum. Samples were placed on aluminium stubs and sputter coated with gold before being analysed. The obtained SEM images were used to measure the size of the microcapsules using Image J software.

2.5.3 Particle Size Distribution

The particle size distribution of the electrosprayed microcapsules were obtained by laser diffraction (Mastersizer®, Malvern), after their dispersion in distilled water, using 1.59 as refractive index of the standard material polystyrene latex.

2.6 Resistance to gastrointestinal media

2.6.1. Preparation of simulated gastric and intestinal juices

The simulated juices were prepared adapting methods described by Charteris, Kelly, Morelli, & Collins (1998) and Michida et al. (2006). Briefly, simulated gastric juices (SGJ) were prepared by suspending pepsin (P7000, 1:10,000) in sterile sodium chloride solution (0.5 %, mass fraction) to a final concentration of 3 g L^{-1} (1,038 U mL^{-1}) and adjusting the pH to 2.0 using concentrated HCl or sterile 0.1 M NaOH. Simulated intestinal juices (SIJ) were prepared by suspending pancreatin USP (P-1500) in sterile

sodium chloride solution (0.5 %, mass fraction) to a final concentration of 1 g L^{-1}, added of 4.5 % (mass fraction) of bile salts and adjusting the pH to 8.0 using sterile 0.1 M NaOH. Both solutions were filtered for sterilization through a 0.22 µm membrane.

2.6.2 Cell tolerance test towards gastrointestinal media

The tolerance of free and microencapsulated cells of *L. plantarum* BL011 on simulated gastric and intestinal juices was determined using the adapted method described by Charteris et al. (1998). The tests were performed using a series of sterile 15 mL Falcon tubes, one for each sample (see times of sampling below) of 0.4 mL of the suspension of either microencapsulated or free cells, added of either 1.8 mL of SGJ or SIJ, gently mixed and incubated for 120 min at 37 ºC ± 1 ºC. The control for these tests was done by incubating 0.4 mL of either free or microencapsulated cells in 1.8 mL sterile sodium chloride solution (0.5 %, mass fraction) under the same conditions. After the addition of samples to SGJ and SIJ, the pH was adjusted to 2.0 or 8.0, respectively, using sterile 0.1 M NaOH or concentrated HCl. Aliquots of 1 mL were collected at 0, 30, 60, and 120 min (for all trials) for the determination of total viable counts using the pour-plate assay.

2.7 Viability of *L. plantarum* BL011 microencapsulated

The viability of microencapsulated cells was analysed using fluorescence microscopy (spectral confocal microscope FV1000, Olympus Optical, Tokyo, Japan). The cells were stained using fluorescein diacetate (FDA) following the method described by Corich, Soldati, & Giacomini (2004). The sterile solutions 1 and 2 were inoculated with cells and were electrosprayed in the dark to preserve the fluorescing FDA dye. Samples were collected on microscope slides for fluorescence microscopy

imaging analysis. The viability of cells was also determined using the pour-plate assay. This test was conducted to determine the viability of cells in the spray solution prior to electrospraying and for storage viability. Briefly, 1 mL of inoculated spray solution was diluted and plated using selective *L. plantarum* differential medium described by Bujalance, Jiménez-Valera, Moreno, & Ruiz-Bravo (2006), and incubated at 37 ºC ± 1 ºC. The viability of *L. plantarum* BL011 in spray solutions and microencapsulated was determined as log (CFU mL^{-1}). The viability assay was performed for samples at intervals of time of 0, 1, 3, 5, 7, 14, and 21 number of days stored at 4 ºC ± 1 ºC.

2.8 Solubilization of microcapsules

The microcapsules were dissolved using 1 mL of bead suspensions in 9 mL 0.1 M phosphate buffer, pH 7.5, after this solution was stirred for 10 min on a rotatory shaker at 37 ºC ± 1 ºC and 180 rpm. The formed solution was then used to determine the number of viable cells.

2.9 Data analysis

ANOVA and Tukey's mean comparison tests ($p \leq 0.05$) were used to evaluate the data obtained from the tests using the Statistic 7.0 software (Statsoft, Tulsa, USA). All experiments and analyses were run in triplicates.

3 RESULTS AND DISCUSSION

3.1 ALG-based microcapsules characterization

The ALG and ALG-PEC containing *L. plantarum* BL011 were electrosprayed and the microcapsules were collected into a 0.5 M $CaCl_2$ solution. The formation of microcapsules was achieved through the immediate interaction between the COO^- of

ALG or ALG-PEC and Ca^{2+} ions at the surface of the microcapsules, producing a rigid external capsule (Daemi & Barikani, 2012; Khaksar et al., 2014; Paques, van der Linden, van Rijn, & Sagis, 2013). The ALG and PEC powders and their microcapsules spectra are shown in Figure 1.

The FTIR spectrum of ALG (Figure 1a) shows the characteristic absorption bands for sodium alginate. The broad band at the interval between 3,600 – 3,200 cm^{-1} corresponding to the stretching of the O-H; the band at 2,890 cm^{-1} corresponds to the C-H stretching vibration; the bands at 1,601 cm^{-1} and at 1,410 cm^{-1} are related do the asymmetric and symmetric stretching vibration of COO^-, respectively; finally, the band at 1,031 cm^{-1}, corresponds to the C-O-C stretching vibration. ALG structure consists of linear chains of (1,4)-linked β-D-mannuronate and R-L-guluronate residues (Figure 2), which can be characterized by appearance of the C-O-C bonds and COO^- groups in the FTIR spectrum, respectively (Khaksar et al., 2014, Siew & Williams, 2005). Although our results clearly demonstrate the presence of the characteristic bands associated to ALG, the absorption bands were slightly different from those described in previous studies (Daemi & Barikani, 2012; Hosseini et al., 2013; Khaksar et al., 2014). Our assumption upon the differences found between this and preceding researches may lay in the fact that different sources of the polysaccharide were used.

The ALG microcapsules showed some differences in the FTIR spectrum compared to ALG. Although no differences in the band associated to the *asymmetric* stretching vibration of COO^- (1,601 cm^{-1}) were observed, the band corresponding to its *symmetric* stretching vibration was shifted to higher wave numbers (1,424 cm^{-1}) (Figure 1b). This can be associated to the replacement of sodium for calcium ions, because of changes in the charge density, radius, and atomic weight of the cation, as ALG shows affinity to metal ions binding (Daemi & Barikani, 2012). The band corresponding to the

C-O-C stretching vibration shifted to lower wave number (1,028 cm^{-1}) and showed a decreased intensity. According to the literature, these changes suggest a partial covalent bonding between calcium and oxygen atoms from C-O-C bond (Hosseini et al., 2013). Moreover, the broad band at 3,600 – 3,200 cm^{-1} region showed a significant decrease in its intensity, associated to the participation of OH and COOH in the chelation process with Ca^{2+} (Daemi & Barikani, 2012).

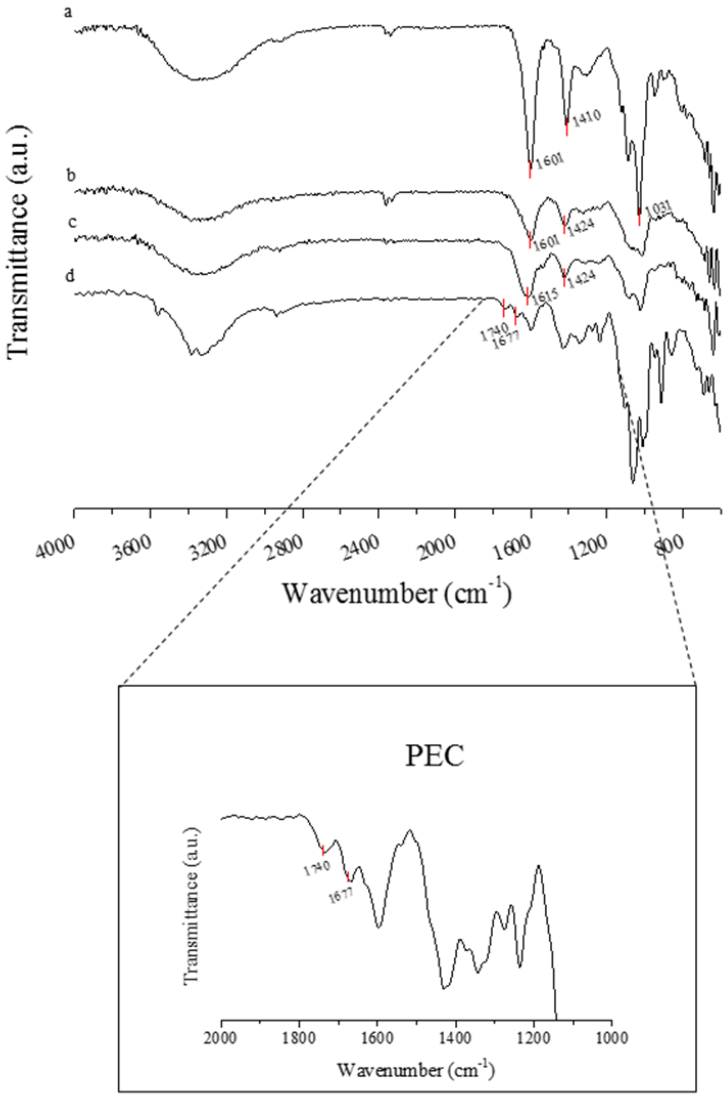

Figure 1. ATR-FTIR spectra of (a) ALG powder, (b) ALG microcapsules, (c) ALG-PEC microcapsules and, (d) PEC powder. The insight in the figure highlights the main bands signed to PEC powder, which appeared in very small intensity.

The FTIR spectrum of PEC (Figure 1d) shows the characteristic bands associated to low-methoxyl pectin, such as the band at 1,740 cm^{-1}, which corresponds to the stretching of the C=O bond of the ester carbonyl group, and at 1,677 cm^{-1}, caused by the stretching vibration of COO^- (Kyomugasho, Christiaens, Shpigelman, Van Loey, & Hendrick, 2015). Pectin is a general term for a family of covalently linked galacturonic acid polysaccharides, partly esterified with methanol (Rinaudo, 2006; Thakur, Singh, Handa, & Rao, 1997). More specifically, its chemical structure consists mainly of linear chains of (1,4)-linked R-D-galacturonic acid residues (Figure 2), where approximately 80 % of the acid groups are in the form of methyl esters (Siew & Williams, 2005).

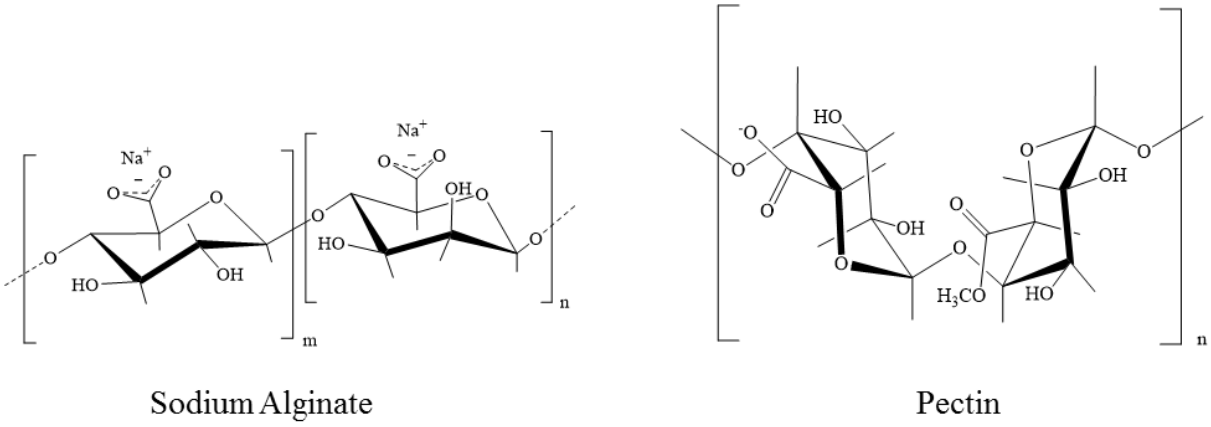

Figure 2 - Chemical structures of sodium alginate and pectin (Adapted from Siew and Williams, 2005, and Thakur et al., 1997).

We can observe in Figure 1c that the ALG-PEC microcapsules spectrum shows an absorption band at 1,615 cm^{-1}. The band presents a broad shape and corresponds to the overlapped bands of carboxyl groups from both ALG and PEC. In addition, this band is shifted to higher wave numbers when compared to ALG spectrum (1,601 cm^{-1}), and shifted to lower wave numbers when compared to PEC spectrum (1,677 cm^{-1}). As mentioned before, the shift of COO^- bands to different wave numbers are associated to the complexation of Ca^{2+} in the carboxyl groups of the polysaccharides (Daemi &

Barikani, 2012). Moreover, the band at 1,740 cm^{-1} region associated to the C=O bond in PEC is not observed. We assumed that the C=O bond deprotonated in the acidic medium and participated of the Ca^{2+} complexation. Both ALG and PEC show specific ion binding characteristics and affinity for alkaline earth metals, like Ca^{2+} (Braccini, Grasso, & Pérez, 1999; Siew & Williams, 2005). It has been demonstrated that the polyguluronate (from ALG) and polygalacturonate (from PEC) chains enable coordination of the Ca^{2+} ion with several oxygen atoms from consecutive residues facilitating high calcium specificity (Braccini et al., 1999). This complexation locates the cation sandwiched within the dimer on specific sites along each of the surfaces ALG and PEC resulting in a phenomenon commonly referred to as the "egg-box model" (Siew & Williams, 2005).

The properties of ALG-based solutions can give us some insights in order to better understand the interactions among ALG, PEC, and Ca^{2+}, as shown in the Table 1. The ALG-PEC solution presented lower pH compared to ALG. This decrease in the pH is associated to the acidic nature of pectin (pK_a ~ 3.5, Rinaudo, 1996). Due to the presence of uronic acid units, pectins are considered polyelectrolytes and, for this reason, their main characteristic is the pK_a of the carboxylic groups (Rinaudo, 1996). PEC can interact with ALG producing a polyelectrolyte complex in a synergic effect that produces a gel at low pH values. This reaction is able to occur at conditions under which neither ALG nor PEC would otherwise jellify, as for example, the absence of divalent ions (Siew & Williams, 2005). The pH value is a crucial factor for this interaction and values above 4 hinders the gel formation (Shukla, Jain, Verma, & Verma, 2011). At pH 4.7 ± 0.01, we observed a low viscosity value for ALG-PEC solution, when compared to ALG. In this case, the polyelectrolyte complex between

ALG and PEC might not have been enough to arise a gel formation (Siew & Williams, 2005).

The solutions containing *L. plantarum* BL011 also presented lower pH values in comparison to solutions without the probiotic (Table 1). In this case, the low pH values are explained by the lactic acid traces present in the probiotic (De Vries et al., 2006). Especially in the case of ALG with *L. plantarum* BL011, where the pH reduction was more significant, the polyelectrolyte ions were neutralized at low pH, increasing the hydrophobic interactions, thus leading to a significant reduction in viscosity (Berger et al., 2004). We also observed that the solutions conductivity increased as an indirect function of pH and viscosity. This result is related to an increase in the concentration of free ions in the acidic solutions, which improves their mobility and, consequently, the conductivity of the solutions (Xu & Angell, 2003).

Table 1. Properties of the ALG electrosprayed solutions and average diameter of their correspondent microcapsules.

Sample	Viscosity (cP)	pH	Conductivity (mS/cm)	Average diameter of microcapsules (µm)
ALG	949 ± 2	7.80 ± 0.14	5.70 ± 0.71	105 ± 15
ALG with *L. plantarum* BL011	541 ± 2	4.85 ± 0.07	6.40 ± 0.14	111 ± 13
ALG-PEC	123 ± 2	4.70 ± 0.01	8.00 ± 0.01	116 ± 13
ALG-PEC with *L. plantarum* BL011	107 ± 1	4.50 ± 0.01	8.20 ± 0.28	109 ± 15

The SEM images of ALG and ALG-PEC microcapsules, with and without *L. plantarum* BL011, are shown in Figure 3. All the microcapsules presented round and rod shapes in the same population of particles. No significant differences among their average diameters were detected (Table 1) and this was not associated to the solutions properties or to the encapsulation of probiotic in the microcapsules. However, a wide range of diameters was observed in the particle size distribution, but all lying in the

range of μm (Figures 3 and 4). The laser diffraction test provided a more representative result of ALG microcapsules size distributions than does the SEM technique. The results obtained by this method showed no sub-μm particles (< 1 μm) for all samples (Figure 4). Only μm populations were observed for ALG, ALG with *L. plantarum* BL011, ALG-PEC with *L. plantarum* BL011 (10 – 100 μm, 100 – 1,000 μm), and ALG-PEC (7 – 40 μm, 40 – 1,000 μm, and 1,000 – 2,000 μm) (Figure 4).

The electrosprayed ALG and ALG-PEC microcapsules containing *L. plantarum* BL011 presented suitable particle size distribution to be introduced in food products. The typical size range of probiotic containing ALG microcapsules, obtained from commonly used microencapsulation techniques (emulsion, extrusion, and spray drying), are in between 5 μm to 4 mm (Rokka & Rantamaki, 2010). The microcapsules size influences the texture of food and is an important factor for the sensory quality, especially because it can affect the mouth feel properties of some foods (Rokka & Rantamaki, 2010). Ideally, the microcapsules size is such that the sensory quality of the food is not affected, while delivering suitable nutraceutical doses of probiotic (McMaster, Kokott, & Slatter, 2005).

It is important to note that the electrospray technique used in this research lead to the formation of a considerable percentage of smaller capsules than most of the commonly used encapsulation techniques (López-Rubio et al., 2012). Smaller capsules are desirable because they improve texture and optical appearance of food products. Along with the microcapsules shape, it can determine product flow properties, which is considered as an important parameter for food processing as well (McMaster et al., 2005). It has also been suggested that particle size reduction introduces several bio-adhesive improvement factors, including increased adhesive force and prolonged gastrointestinal transit time (Chen, Remondetto, & Subirade, 2006). Furthermore, it can

potentially increase the bioavailability of certain bioactives by enhancing gastrointestinal absorption (López-Rubio et al., 2012).

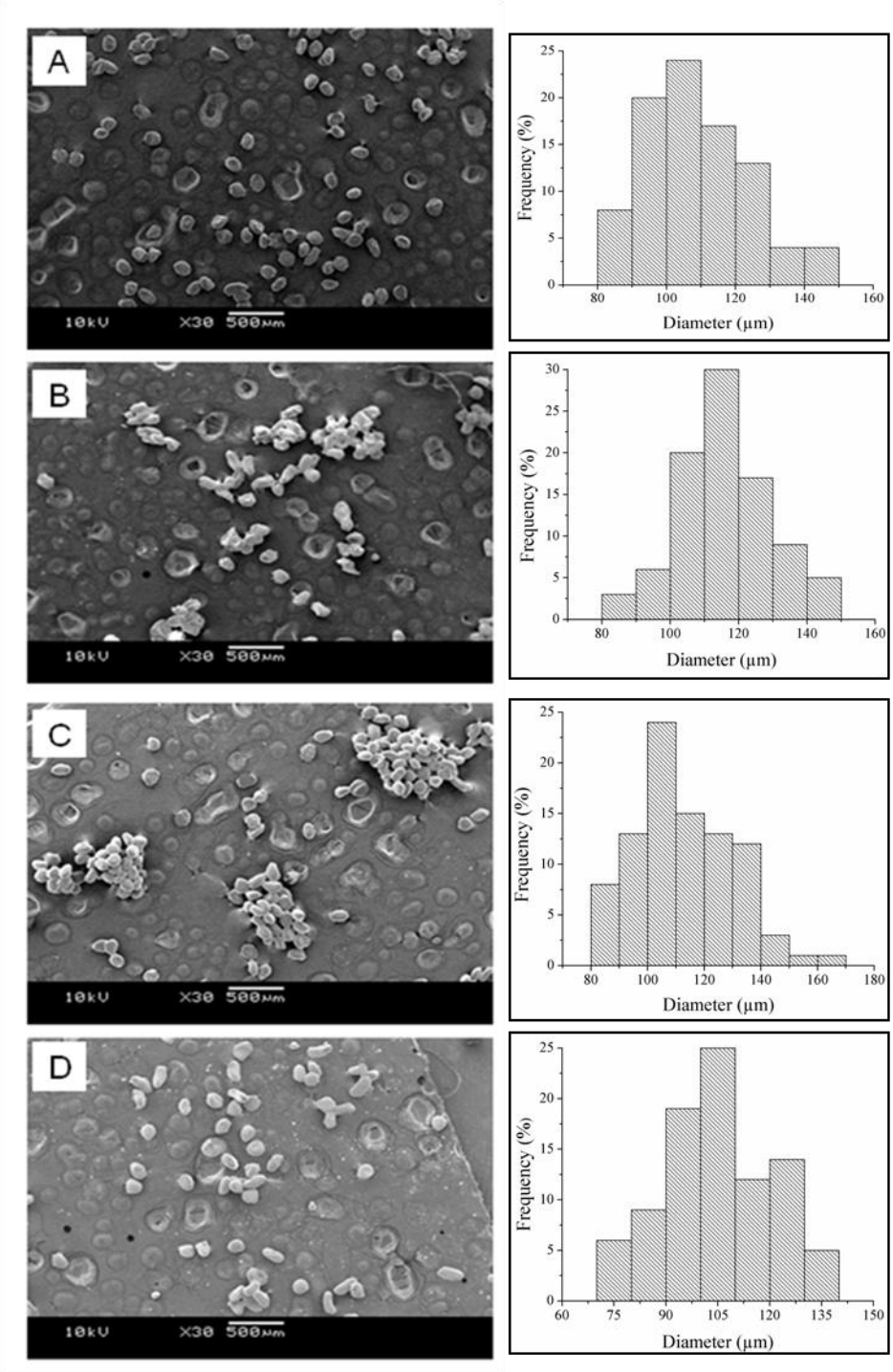

Figure 3. SEM images and capsule size distributions of: (A) ALG-PEC with *Lactobacillus plantarum* BL011; (B) ALG-PEC without *L. plantarum* BL011; (C) ALG with *L. plantarum* BL011; and (D) ALG without *L. plantarum* BL011.

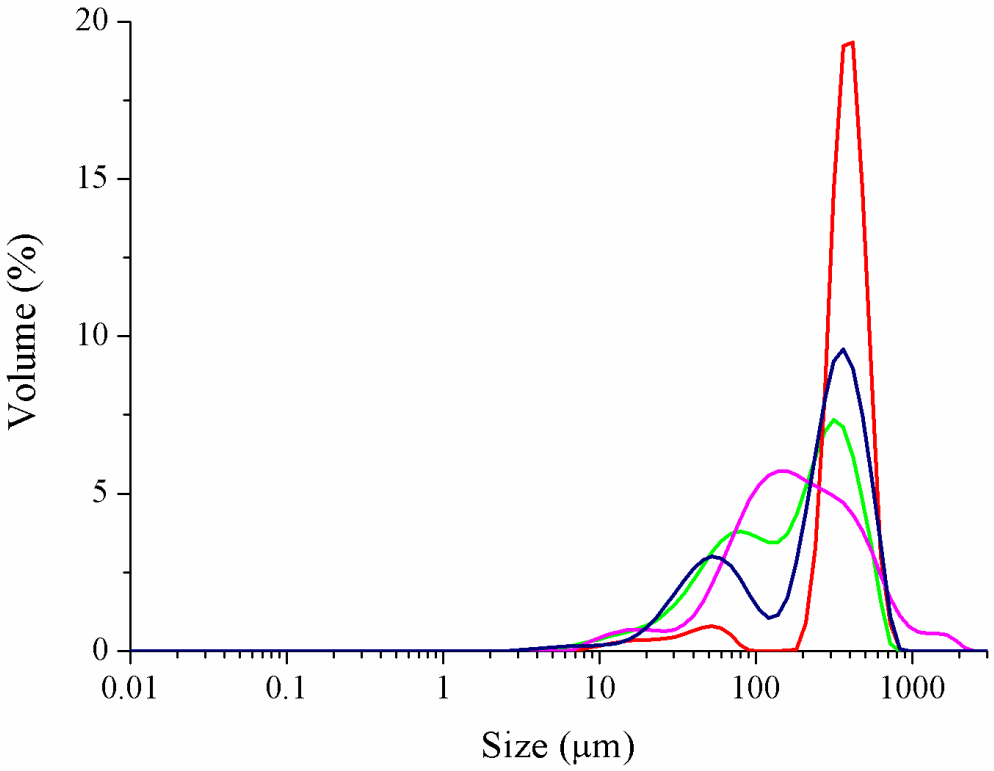

Figure 4. Distribution of the particle size of sodium alginate and citric pectin with and without microencapsulated probiotic. (—) ALG-PEC with *L. plantarum* BL011; (—) ALG-PEC without *L. plantarum* BL011; (—) ALG with *L. plantarum* BL011; and (—) ALG without *L. plantarum* BL011.

3.2 Resistance of electrosprayed-microencapsulated cells of *L. plantarum* BL011 when exposed to gastrointestinal media

One of the most important aspects related to the use of probiotics is their viability in the gastrointestinal system of the host, surviving the action of lytic enzymes and adverse pH. Therefore, is of fundamental importance to test new probiotic formulations concerning their resistance towards gastric and intestinal juices. In Figure 5 are shown the results of the test for the exposition of cells to simulated gastric and intestinal juices. The results show extensive loss of viability of free cells of *L. plantarum* (control) when incubated for 120 min in SGJ and in SIJ, decreasing by 6 and

by 4.2 log CFU mL^{-1}, respectively. In contrast, when cells were microencapsulated in ALG and in ALG-PEC, they showed significant resistances to SGJ and SIJ, decreasing by 2.9 and 2.7 log CFU mL^{-1}, respectively. These results show that the total amount of viable cells in the microencapsulated systems remained within the values required by international recommendations, which determine that probiotics present in food should survive in significant numbers, around 10^6-10^8 CFU mL^{-1} or g^{-1} of product, depending on the strain of LAB (Amine et al., 2014; Anekella & Orsat, 2013).

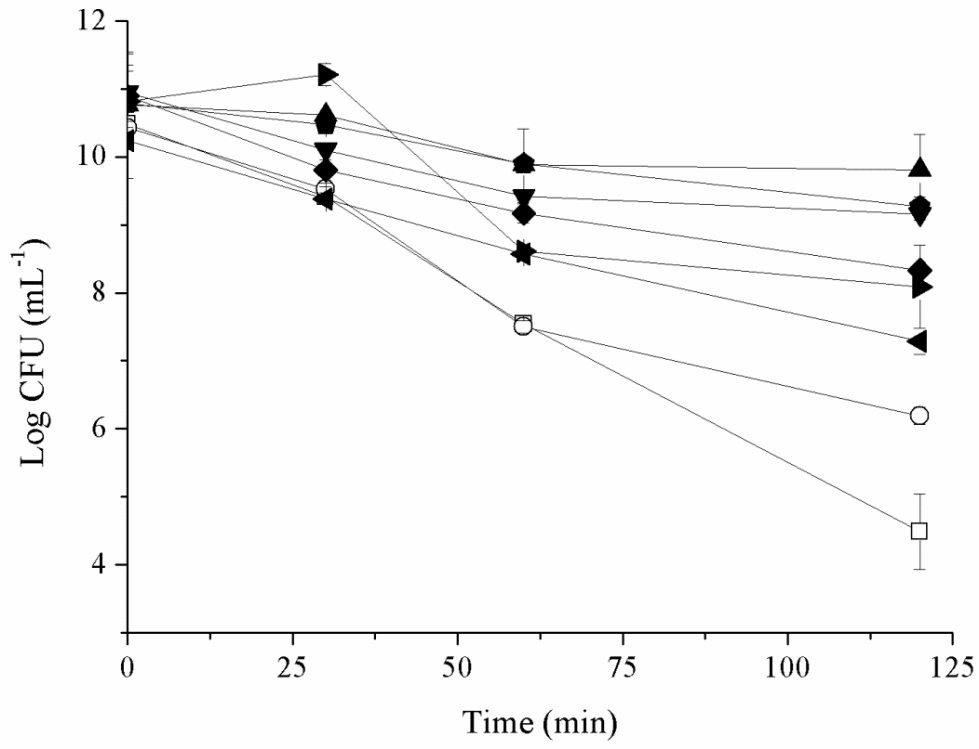

Figure 5. Variation in the number of viable cells of *Lactobacillus plantarum* BL011 when subjected to intestinal (SIJ) and gastric (SGJ) juices. (□) free cells in SGJ; (○) free cells in SIJ; (▲) control ALG; (▼) control ALG-PEC; (►) ALG microencapsulated cells in SGJ; (⬟) ALG microencapsulated cells in SIJ; (◄) ALG-PEC microencapsulated cells in SGJ; (♦) ALG-PEC microencapsulated cells in SIJ.

The results demonstrated that both microencapsulation matrices were effective in protecting cells against SGJ and SIJ, and the technique of electrospraying was suitable to prepare these microcapsules, being a promising alternative for the encapsulation of probiotics (López-Rubio et al., 2012).

The viability results obtained for the exposure to SGJ and SIJ compare well with previous reports on the literature, where different matrixes and immobilization techniques were used. Brinques & Ayub (2011) used the same LAB and the same simulated media and tested the viability of immobilized cells in sodium alginate, chitosan, or citric pectin beads, prepared by conventional methods of entrapment using calcium chloride. The authors found that SGJ produced only a small reduction of viable cells in comparison with the control (free cells), however, the SIJ drastically reduced the cell viability in all matrixes, demonstrating that the conventional calcium chloride-emulsions microencapsulation did not entirely protected the cells.

Martoni et al. (2007) used extruded alginate and poly-L-lysine matrixes to immobilize *L. plantarum* 80 BSH+. The authors reported reductions of 1.09 and 0.6 log CFU mL^{-1} of cell viability under pH 2.5 and 3.0, respectively, when cells were exposed to simulated stomach conditions for 4 h. Decrease in the viability of cells was extreme when they were exposed for 4 h at pH 2.0, reaching 8.98 log CFU mL^{-1} of reduction. At pH 1.5, there were no viable cells after 30 min of exposure.

To overcome these losses of cell viability, techniques that use multi-layers of matrixes have been developed and tested. For instance, Gbassi, Vandamme, Ennahar, & Marchioni (2009) demonstrated that, after 90 min of incubation, three different strains of *L. plantarum* spp encapsulated with calcium alginate, showed total loss of viability. However, when the authors coated the alginate matrixes using whey protein, they observed an increase in survival, showing that the technique was effective for the

protection of the probiotic strains. Nualkaekul et al. (2012) assessed the viability of *L. plantarum* NCIMB 8826 microencapsulated by extrusion in alginate and subsequent coating with two layers of chitosan. When cells were incubated in simulated gastric solution at pH 1.5 for 2 h, a final cell count of 6.2 log CFU mL^{-1} was obtained, showing the protective effect of this technique. The microencapsulation *L. acidophilus* through layer-by-layer self-assembly of chitosan and carboxymethyl cellulose was used to evaluate the survival rate of probiotics under SGJ and SIJ (Priya, Vijayalakshmi, & Raichur, 2011). The authors reported a final viability of 6 log CFU 500 mg^{-1} of encapsulated cells after 120 min exposure when the immobilized beads were coated with three nanolayers of polyelectrolyte, sharply contrasting with results for the uncoated beads, which showed viabilities of only 1 log CFU 500 mg^{-1}. The enhanced survival of cells was attributed to the impermeability and stability of polyelectrolyte nanolayers against pepsin and pancreatin (Priya et al., 2011). In the present study, we found similar results without the use of complex coating techniques.

The reasons for the good results of cell viability in our work might be explained by the fact that the technique of electrospraying can produce microcapsules without the need of using excessive heat. Furthermore, the microcapsules can support high number of cell loads, and can be applied to produce core-shell microcapsules (Laelorspoen, Wongsasulaka, Yoovidhyaa, & Devahastina, 2014; López-Rubio et al., 2012). The entrapped cells should be able to stay within the coreof microcapsules, in a way that the protection capability of the microcapsules is maximized. Finally, the release mechanism of the microcapsules can be manipulated via the proper design of the shell matrix of the microcapsules (Laelorspoen et al., 2014; López-Rubio et al., 2012).

3.3 Viability of electrosprayed-microencapsulated cells of *L. plantarum* BL011 under cold storage

Another important aspect of food products containing probiotics is the cell viability along time during storage because this is a common operation in commercialization. In this work, we tested the variation in viability of electrosprayed-microencapsulated *L. plantarum* BL011 when stored under refrigeration temperature (4 ºC ± 1 ºC) for a period of 21 days (Figure 6). The viability of *L. plantarum* BL011 resuspended in the spray solution (but not microencapsulated) was also measured to assess the effect of the electrospraying technique itself on cells. As it is shown in Figure 6, the reductions in viability of electrosprayed-microencapsulated *L. plantarum* BL011 were not significant considering the practical application (survival in products), with cell viability kept above 9.3 log CFU mL^{-1} and 8.6 log CFU mL^{-1} of their initial values after 21 days of storage for ALG and ALG-PEC systems, respectively. In contrast, free cells resuspended in the spraying solution quickly lost viability, with less than 6 log CFU mL^{-1} remaining after 8 days of storage and only 1.2 log CFU mL^{-1} of cells still viable at the end of the 21 days. During the revision of the technique of electrospraying we were concerned about the possible effects of the change in the osmotic environmental pressure surrounding cells caused by the rapid evaporation of water upon electrospraying, the high voltage applied to the material and the shear mechanical stress during electrospraying. None of these effects was involved in reducing cell viability, again confirming the potential of this technique for the immobilization of LAB.

These results are comparable to results reported for other probiotics encapsulated using the electrospinning technique and different matrixes. Fung et al. (2010) used the electrospinning technique for the encapsulation of *L. acidophilus* in fractions of soluble dietary fibres. The authors obtained significant survival of probiotics when stored for 21

days at 4 ºC, (78.6 to 90 %), and the thermal resistance of the encapsulated probiotic suggested a possible thermal protection of cells when delivered in heat-processed foods.

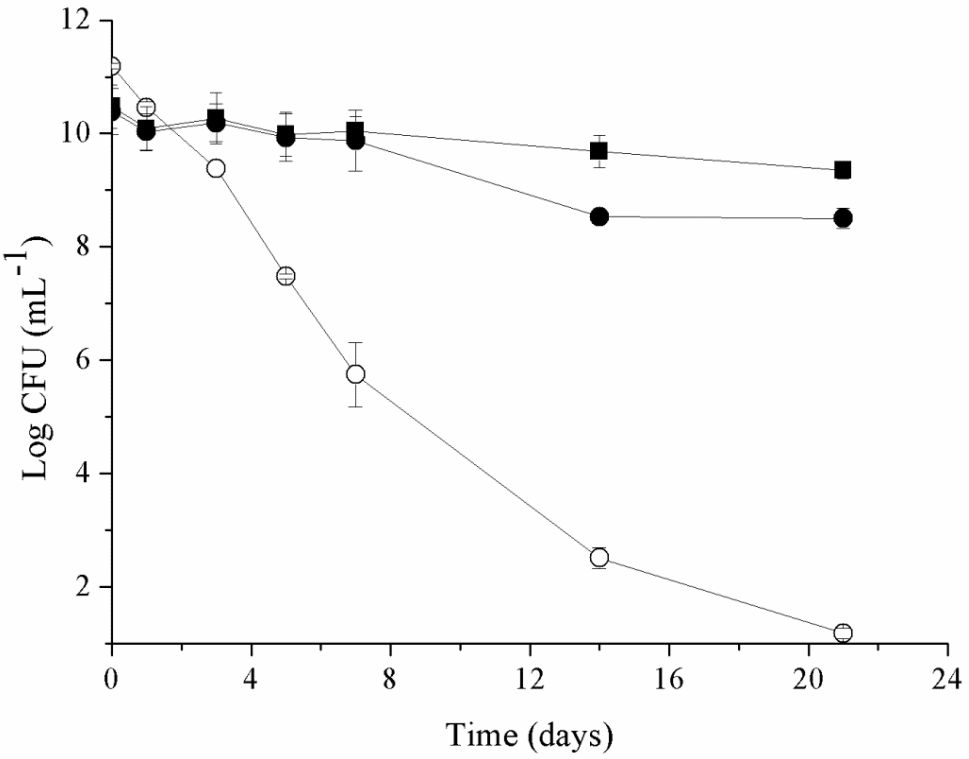

Figure 6. Viability of *Lactobacillus plantarum* BL011 in ALG (■), ALG-PEC (●) and free cells (○) when exposed to refrigeration temperature over time.

Again, the results obtained for the preservation of cell viability in our work compares well with the viability of cell systems using traditional techniques of conservation. For instance, Capela, Hay, & Shah (2006) reported on the cell survival after processing and storage of *L. acidophilus*, *L. casei*, *L. rhamnosus*, and *Bifidobacterium* spp., microencapsulated in sodium alginate beads and using cryoprotectants (Unipectine RS 150 and Satialgine GCF 639), added to yoghurt and freeze-dried yoghurt. The microencapsulated cells had their viability improved in freeze-dried yoghurt after 6 months of storage at 4 ºC (8.93 to 8.67 log CFU g^{-1}) and at

21 ºC (8.93 to 6.79 log CFU g^{-1}). In contrast, the unprotected free cells, showed final viabilities, at the end of the storage time, of 8.40 log CFU g^{-1} (4 ^{o}C) and 6.19 log CFU g^{-1} (21 ^{o}C).

To further confirm that cells were effectively microencapsulated and kept their viability after electrospraying, spheres of ALG and ALG-PEC were stained with FDA and inspected under optical micrograph, results that are shown in Figure 7. It is possible to visualize the presence and the even distribution of viable cells into microcapsules (green dots) for both ALG and ALG-PEC. Furthermore, we noticed no morphological modifications of the cells.

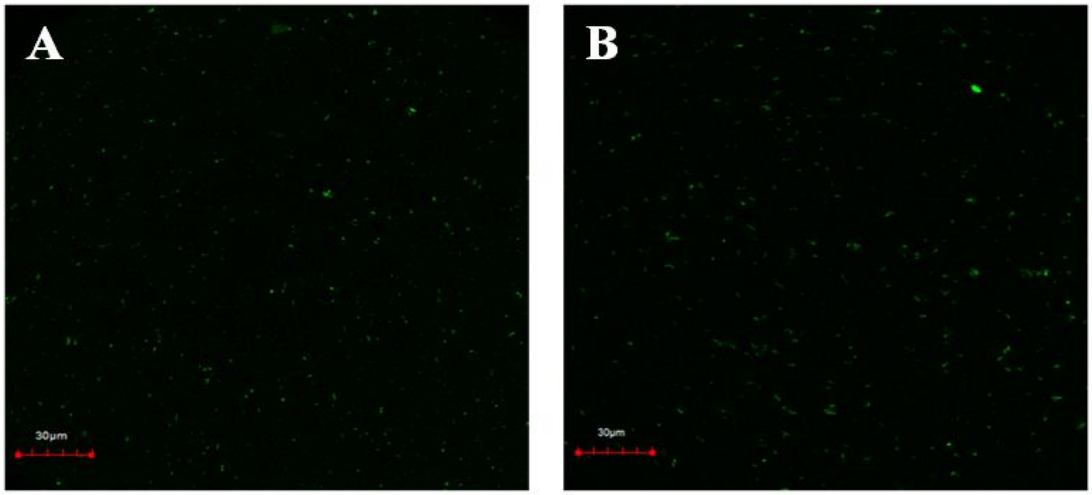

Figure 7. Optical micrograph of FDA-stained *Lactobacillus plantarum* BL011 encapsulated within electrosprayed microcapsules in ALG-PEC 4 % (A), and ALG 3 % (B) solutions. Fluorescence illumination revealed live probiotic cells within the capsules.

4. CONCLUSION

ALG and ALG-PEC-based microcapsules of probiotic *L. plantarum* BL011 were successfully produced using the electrospray technique. In order to assure the protection

of the probiotic in the microcapsules, the ALG and ALG-PEC solutions were electrosprayed into a 0.5 M $CaCl_2$ solution producing microcapsules with a cross-linked surface. The FTIR analysis confirmed the interaction by a metal complexation between Ca^{2+} and the deprotonated oxygen through shifts in the bands corresponding to COO^- groups of both ALG and ALG-PEC microcapsules. The obtained round and rod shaped microcapsules showed average diameter and particle size distribution in the interval of μm. These results were not associated with either the presence of PEC or probiotic cells. The microcapsules produced a substantial improvement of cell viability when compared to free cells. Electrosprayed microcapsules demonstrated the ability to prolong *L. plantarum* BL011 survival when exposed to SGJ and SIJ and during storage at 4 ºC for 21 days. These results suggest that electrosprayed probiotics might be used in food formulations to prolong their beneficial effects for the host organism.

ACKNOLEDGMENTS

The authors wish to thank CNPq and CAPES (Brazilian research funding agencies) for their financial support and scholarships for this research.

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CAPÍTULO V – VIABILITY AND ALTERNATIVE USES OF A DRIED POWDER, MICROENCAPSULATED *LACTOBACILLUS PLANTARUM* WITHOUT THE USE OF COLD CHAIN OR DAIRY PRODUCTS

Este artigo foi submetido ao periódico ***Food & Fuction***

Viability and alternative uses of a dried powder, microencapsulated *Lactobacillus plantarum* without the use of cold chain or dairy products

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Abstract

We developed a dried powder of microencapsulated *Lactobacillus plantarum*, a probiotic bacterium, to be added directly into food or food preparations without needing cold storage, allowing its use at 25 ºC. Cells were microencapsulated in calcium alginate using the technique of electrospray. Cell viability during six months of storage, cell survival in orange juice and in simulated gastric and intestinal juices, product contaminations, and the sensory acceptance test of the formulated product were analyzed. Results show that viability of microencapsulated cells was high after six months of storage, keeping 7 log CFU g^{-1} product of its initial value. The viability of cells when formulated in the natural orange juice and after incubation for 120 min in simulated gastric and intestinal juices, presented significant resistance in both environments, decreasing by 2.4 and 1.3 log CFU mL^{-1}, respectively. The microbiological analysis performed before and after the six months storage time did not show any contaminations by pathogens. The formulated orange juice containing *Lactobacillus plantarum* obtained high sensory acceptance (> 88 %) in the sensory analysis. This technology seems to be an interesting alternative for the consumption of viable probiotic bacteria in non-dairy products and without requiring refrigerated storage.

Keywords: *Lactobacillus plantarum*; electrospray; sodium alginate; powder food; sensory analysis.

1 Introduction

The market offer of foods containing probiotics that could be more easily handled by consumers would provide several advantages, including transportation without the need for cold storage and enhanced microbiological safety.[1] Probiotic bacteria and other lactic acid bacteria (LAB) are, in most cases, added as functional ingredients to dairy products such as fermented milks, yogurts, cheese, and ice creams.[2-7] However, because of increasing alternative dietary habits, such as veganism, vegetarianism, and concerns about allergies caused by dairy products, there has been an increasing demand for probiotics by non-dairy consumers. Lactose intolerance and the cholesterol content of dairy products can be considered two major drawbacks related to the consumption of fermented or non-fermented dairy foods.[8-10] Concerning food processing and distribution, many products have the disadvantage of requiring a cold chain of storage and transportation, hindering a wider inclusion of food supplemented with probiotics to the general population, especially in poor countries. Therefore, there is a growing demand by consumers for food products and probiotics that would not require refrigeration. In the case of probiotics, the costs of keeping a cold chain could be greatly reduced, or even eliminated, by the development of dried powders containing these LAB, which could also provide better stability/viability of cells over traditional marketed products.[11]

The viability of probiotic cells should be kept during manufacture, storage, and the passage through the gastrointestinal tract of the host in order to exert their health benefits.[12,13] Probiotic bacteria should survive in significant numbers of at least 10^6 to 10^8 CFU g^{-1} in foods, values that change from strain to strain.[9,14-16] Therefore, in order to improve the viability of probiotics in products and increase their survival to adverse conditions, techniques of cell encapsulation have been devised and used in model

functional foods.[17] The technique of the electrospray microencapsulation can be used to produce small gel capsules, ranging from the micro to the sub-micro sizes, which do not require high operational temperatures to be obtained.[18-20] It has been suggested that this technique could improve the viability of some immobilized LAB to be used as probiotics.[20,21]

Bacteria of the genera *Lactobacillus* and *Bifidobacterium* are classified among the main probiotics considered safe for use in food and feed.[22] Therefore, there is a great interest in screening for new potentially probiotic strains of LAB, such as *L. plantarum.*

In the light of these considerations, we devised the idea of developing a dried powder of microencapsulated *Lactobacillus plantarum*, a potentially probiotic bacteria, using the technique of electrospraying of Ca-alginate biopolymer and testing this system directly into food, in this case orange juice. We tested the survival of cells during storage time and under incubation in simulated gastric and intestinal juices. Finally, a sensory analysis test was conducted to evaluate the acceptance of a formulated orange juice containing the microencapsulated *Lactobacillus* preparation.

2 Materials and Methods

2.1 Materials

Lactobacillus plantarum strain BL011 was used in this research. This strain was isolated from Serrano cheese, and it was characterized and described elsewhere.[23] The strain is kept in the Microbiology Culture Collection of BiotecLab (UFRGS, Porto Alegre, Brazil). The strain was identified in the DSMA Laboratory (Mogi das Cruzes, SP, Brazil), by comparing the 16S rRNA amplicon sequences with GenBank databases (access number AB5989861), which showed 100 % homology with *Lactobacillus plantarum* genus and species. Stock cultures were kept at 20 % (volume fraction) of a

glycerol suspension, frozen at -22 °C ± 1 ºC. Food grade sodium alginate was used in the microencapsulation process (Gastronomylab, Brasília, Brazil). Freshly pressed natural orange juice (Naturale, Porto Alegre, Brazil), without the addition of sugar or water and without any thermal or chemical treatments, was purchased in local markets in the day of the experiments (Porto Alegre, Brazil). Other reagents were purchased from Sigma-Aldrich (St. Louis, USA).

2.2 Inocula preparation

The cultivation of *L. plantarum* BL011 cells was carried out by inoculating 1.5 mL of glycerol cell stocks in Erlenmeyer flasks (2000 mL), filled with 400 mL MRS[24] and grown for 12 h at 37 °C ± 1 °C in a rotatory shaker (MA 830 Marconi, Piracicaba, Brazil) at 180 rpm, to a final optical density (OD) of 1.0 at 600 nm. The cells were harvested by centrifugation (3500 *g*, Hitachi CR21 E, Tokyo, Japan) and the pellet resuspended directly into the cultivation broth (150 mL), and added to the bioreactor (B. Braun Biotech International, Melsungen, Germany, 2000 mL), filled with 1350 mL of this same medium. The cultivation broth was formulated to contain 40 g L^{-1} of sugars (naturally occurring in the liquid acid residue of soybean), 15 g L^{-1} of raw yeast extract, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 0.04 g L^{-1} $MnSO_4 \cdot H_2O$. Culture conditions were 25 °C, pH 5.5 ± 0.2, agitation 200 rpm, and 4.5 vvm of aeration. After 72 h of cultivation, cells were harvested by centrifugation (3500 *g*) for 15 min at 4 °C ± 1 °C and immediately added to solution of sodium alginate.

2.3 Microencapsulation of *L. plantarum* by electrospraying

The probiotic was microencapsulated using the technique of electrospraying, using an in-house made electrospinning equipment.[21] The microencapsulation

biopolymer was Ca-alginate and the general procedure of the technique was as follows. A solution of sodium alginate (3 %, mass fraction) was mixed with 10.5 log CFU mL^{-1} cells and the preparation was introduced into a 1 mL plastic syringe and electrosprayed under steady flow-rate of 2 mL h^{-1} using a stainless-steel needle of 0.06 mm of diameter. The applied voltage was 24 kV and the distance between the needle tip to the collector was 15 cm. The formed microspheres were collected into a beaker containing a solution of calcium chloride ($CaCl_2$, 0.5 M), and the formed beads were immediately freeze-dried. The samples were frozen at -80 °C in sterilized 15 mL flasks (Ultrafreezer Cold Lab, Piracicaba, Brazil) for 18 h, and lyophilized (Lyophilizer LIOTOP L101, Liobras, Brazil) at a pressure of 100 μHg and -50 °C, for 24 h in order to obtain the *L. plantarum* microencapsulated powder (LPMP). LPMP was stored in the same flasks used for freeze-drying and stored at 25 ºC ± 1 °C until use.

2.4 Optical microscopy

Optical microscopy images were taken using a digital microscopy system Olympus CX31 equipped with a MSHOT digital microscope camera MD90.

2.5 Scanning Electron Microscopy (SEM)

The morphologies of the microcapsules were analyzed under scanning electron microscopy using a SE-microscope (model JSM 6060, Tokyo, Japan), operating at an accelerate voltage of 10 keV (kiloelectron-volts). Samples were placed on aluminum stubs and sputter coated with gold before being analyzed, following the methodology described by Najafpour *et al.*[25]

2.6 Cell viability at room temperature

Cell viability in the microencapsulated powder (LPMP) was analyzed throughout 6 months of storage at 25 ºC ± 1 ºC. The LPMP spheres were solubilized using 0.1 g of bead suspensions in 0.9 mL 0.1 M phosphate buffer, pH 7.5, added into an 2 mL Eppendorf and stirred for 10 min on a rotatory shaker at 37 ºC ± 1 ºC and 180 rpm. This preparation was used to determine the number of viable cells, plated using selective *L. plantarum* differential medium and incubated at 37 ºC ± 1 ºC, following the method described by Bujalance *et al.*[26]

2.7 Viability of microencapsulated *L. plantarum* under incubation in simulated gastric and intestinal juices

The viabilities of microencapsulated cells as LPMP added into natural orange juice (10 log CFU 200 mL^{-1} of juice), and were evaluated after incubation under simulated gastric juice and simulated intestinal juice (SGJ and SIJ), following procedures described by Charteris *et al.*[27] The tests were performed using a series of sterile 15 mL Falcon tubes, one for each sample (see intervals of sampling below) of 0.4 mL of orange juice suspension of either microencapsulated or free cells, added of either 1.8 mL of SGJ or SIJ, gently mixed and incubated for 120 min at 37 ºC ± 1 ºC. After the addition of SGJ and SIJ to samples, the pH of them were adjusted to 2.0 and 8.0, respectively, using sterile 0.1 M NaOH or concentrated HCl. Aliquots of 1 mL were collected at 0, 30, 60, and 120 min (for all trials) for the determination of total viable counts using the pour-plate assay, according to the method described by Bujalance *et al.*[26]

2.8 Microbiological analysis

The presence of pathogens and other microorganisms in LPMP samples was analyzed immediately after the freeze-drying procedure and after six months of storage at 25 ºC ± 1 ºC. The microorganisms that were investigated were *Bacillus cereus*, Coliforms, Coliforms 45 ºC, *Salmonella* spp, and *Staphylococcus* coagulase positive.[28-30]

2.9 Sensory analysis

The sensory evaluation of the natural orange juice samples was conducted using an acceptance test performed by 90 untrained panelists, 71 women and 19 men between 18 and 60 years old, who were selected after positively answering that they were potential fruit juice consumers. In order to obtain a final cell concentration of 10 log CFU L^{-1} of juice, 0.5 g of LPMP was added to 1 L of juice, mixed well using a spoon and immediately provided in 50 mL cups, containing approximately 30 mL of juice (with or without cells), coded using three-digit random numbers.

The acceptance of attributes such as appearance, color, odor, taste, viscosity, and overall acceptability were evaluated using a hedonic scale of 9 points. The consumers were asked about purchase intention of juices.[31] The study was ethically evaluated and the permission to carry on was awarded by the University Ethical Committee (Protocol #908972, Federal University of Rio Grande do Sul, Porto Alegre, Brazil) and the participants were informed of every detail of the scope of the present research.

Sensory analysis data were evaluated by analysis of variance (ANOVA) and Tukey test at significance level of 0.05 using the software Statistica 10.0 (Statsoft, Tulsa, USA).

3 Results and Discussion

3.1 Morphology of the microcapsules before and after freeze-drying

The morphology of microcapsules must be analyzed in order to verify whether cells were effectively microencapsulated in large numbers, and to make sure that the spheres were of functional size to be added into food formulations. Figure 1 depicts the phase-contrast microscope images of *L. plantarum* BL011 within the electrosprayed Ca-alginate microcapsules before undergoing the freeze-drying process. The presence of cells confirms that this technique is effective for microencapsulating this *Lactobacillus*. In Figure 2 are depicted the LPMP after microencapsulation and freeze-drying, showing the external surface of beads (Figure 2 A), the inner structure of the microcapsules (Figure 2 B), and the entrapped *L. plantarum* BL011 in capsules of calcium alginate (Figure 2 C).

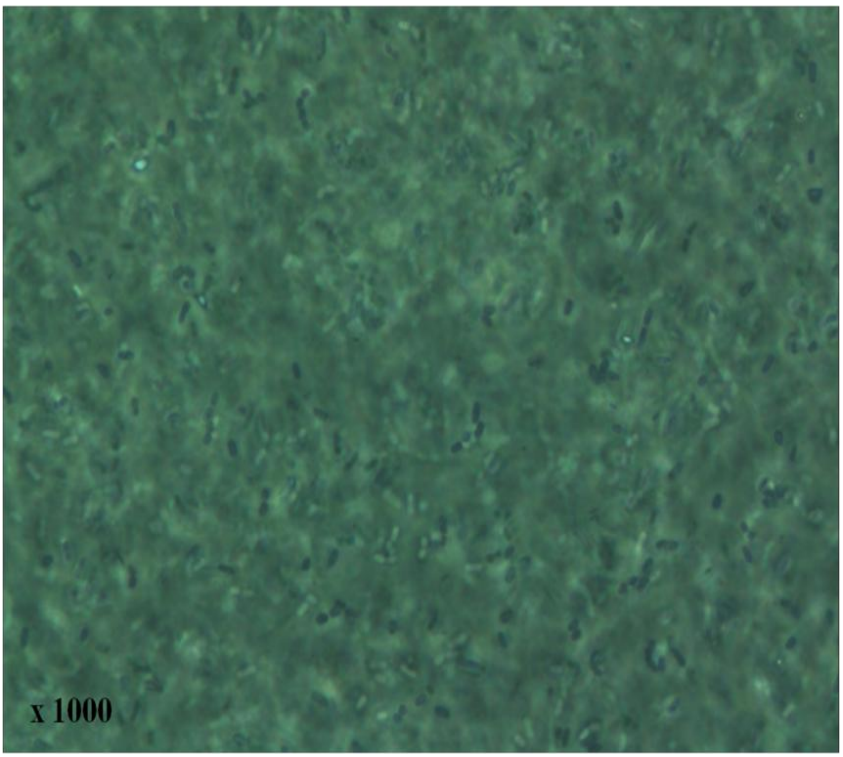

Figure 1 Phase-contrast images of *L. plantarum* BL011 microencapsulated in sodium alginate by electrospraying.

The microcapsules sizes were controlled by the needle diameter and the spheres of each formulation had a diameter ranging from 100 to 300 μm, as demonstrated by the SEM analysis of the shapes of the freeze-dryed capsules shown in Figure 2 (A, B and C). Most of the capsules were predominantly spherical, although some were found to be elongated or irregular. Although most of the microcapsules surfaces appeared smooth (Fig. 2 A and B), part of them showed a rough surface (Fig. 2 C), compatible with the shrinkage occurring during the drying process.[32,33]

The inner structure of the microcapsules (Fig. 2 C) suggests a very compact texture, probably as a consequence of the drying process and resulting from water evaporation. The interior of the capsules, revealed by fracturing, shows groups of entrapped bacteria (arrows), on the surface of alginate microcapsule.[33]

The general observation of the SEM analysis confirms that the microcapsules containing *L. plantarum* BL011 had an adequate size for addition in food products. The spheres ranging from 100 to 300 μm obtained in our work compare well with values obtained when using other microencapsulation techniques, which produced sphere sizes in the range of 5μm to 4 mm.[34] The relatively small sizes of the microcapsules influence the texture of food, optical appearance, and these aspects may affect the sensory quality of the food.[34,35]

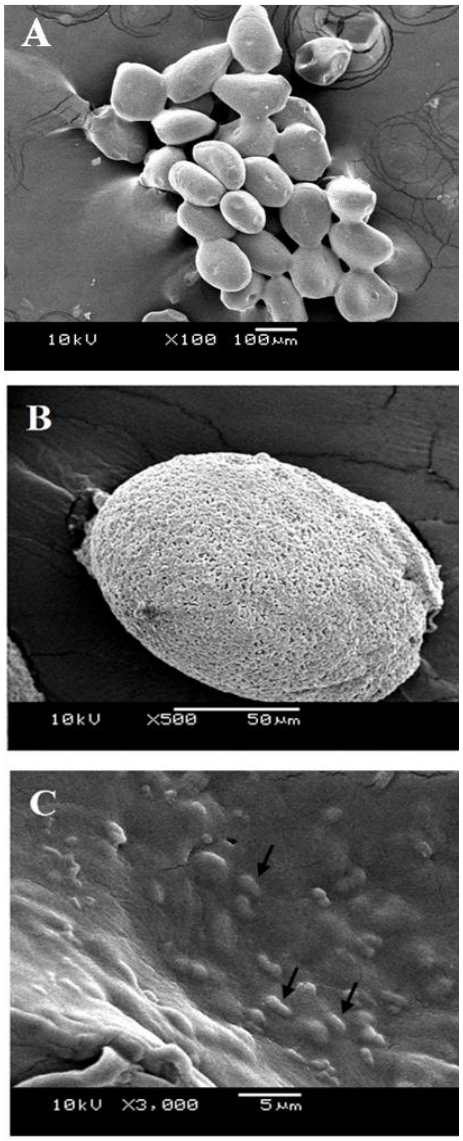

Figure 2 SEM pictures of LPMP after microencapsulation and freeze-drying: (A and B) external surface of beads; (C) inner structure of capsules showing entrapped *L. plantarum* BL011 in capsules of calcium alginate.

3.2 Viability of LPMP during the storage at room temperature

Another important aspect concerning food products containing probiotics is the viability of cells during the time of storage or the shelf life. We tested the variation of viability of LPMP when these were stored under 25 ºC for a period of 6 months, and results are depicted in Figure 3. We can observe a reduction in the viability in the

LPMP, with data showing a loss of 3.5 log CFU g^{-1} after 6 months of storage. However, the final concentration of 7 log CFU g^{-1} of viable cells is within the recommended concentrations of 10^6 to 10^8 CFU g^{-1} in foods.[9,14,16]

Although strains vary from study to study, our results compare well with reports in the literature for tests of LAB survival in different food formulations and environments. For instance, Ying *et al.*[13] reported on the stability of *L. rhamnosus* GG for 5 weeks storage at 25 °C, obtaining a viability of 7 log CFU g^{-1}. Capela *et al.*[36] reported on the cell survival of microencapsulated *L. acidophilus*, *L. casei, L. rhamnosus*, and *Bifidobacterium* spp., in sodium alginate added of cryoprotectants (Unipectine RS 150 and Satialgine GCF 639), formulated in yoghurt and freeze-dried yoghurt. The microencapsulated cells had their viability improved in freeze-dried yoghurt compared against the free cells after 6 months of storage at 4 ºC (8.93 to 8.67 log CFU g^{-1}) and at 21 ºC (8.93 to 6.79 log CFU g^{-1}).

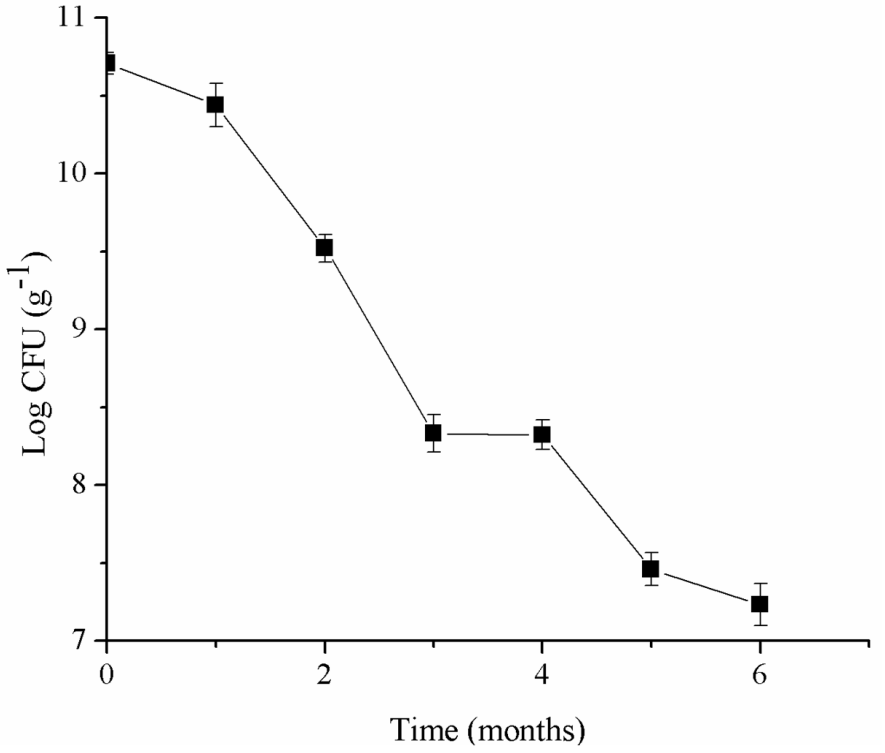

Figure 3 Viability of LPMP exposed to room temperature (25 ºC) during 6 months of storage. Results are the mean of triplicates.

Fung *et al.*[37] used the electrospinning technique to encapsulate of *L. acidophilus* in fractions of soluble dietary fibers. The authors obtained significant survival of probiotics when they stored the cell system during 21 days at 4 ºC, (78.6 to 90 %), and the thermal resistance of the encapsulated probiotic suggested a possible protection of cells when delivered in heat-processed foods.

Finally, Jalali *et al.*[38] studied the preparation of oral capsules of *L. paracasei* subsp. *tolerance* and *L. delbrueckii* subsp. *bulgaricus* obtained by freeze-drying, and analyzed the effects of the addition of cryoprotectants skim milk, trehalose, and sodium ascorbate on the viability of cells over 3 months of storage at 23 ºC. The maximum survival was observed using media containing 6 % skim milk, 8 % trehalose, and 4 % sodium ascorbate, with survival of 10.9 log CFU g^{-1} for *L. paracasei* subsp *tolerance*, and 9.6 log CFU g^{-1} for *L. delbrueckii* subsp. *Bulgaricus*, representing 76 % and 72 % in relation to the initial cell numbers in samples, respectively.

3.3 Cell viability of LPMP incubated under gastrointestinal media

Figure 4 shows the results of the test for the cell viability of LPMP when added to natural orange juice and incubated under simulated gastric and intestinal juices. The results show good cell survival of LPMP after 120 min incubation time under SGJ and SIJ, cell numbers decreasing by 2.4 and 1.3 log CFU mL^{-1}, respectively. Final cell survival was within the parameters required by international recommendations, which determine that probiotics present in food should survive in significant numbers, around 10^6 to 10^8 log CFU mL^{-1} or g^{-1} of product, depending on the strain of LAB.[7,14,16]

Brinques and Ayub[5] analyzed the survival of *L. plantarum* BL011 in gastric and intestinal juices after immobilizing cells by emulsification in Ca-alginate, chitosan, or citric pectin. Their results showed that the intestinal juice causes a small change in the

viability of cells in relation to the control. However, the gastric juice drastically reduced the viability of cells in all matrixes, and the authors concluded that the method of calcium chloride-emulsions microencapsulation offered limited protection to bacteria cells.

Nualkaekul *et al.*[39] reported on the viability of *L. plantarum* NCIMB 8826 microencapsulated by extrusion in alginate, subsequently coated with two layers of chitosan. Simulated gastric solution (pH 1.5) was used to incubate 8.4 log CFU mL^{-1} of cells. At the end of 4 h of incubation, a final survival of 5.2 log CFU mL^{-1} was obtained, proving the protective effect of this technique.

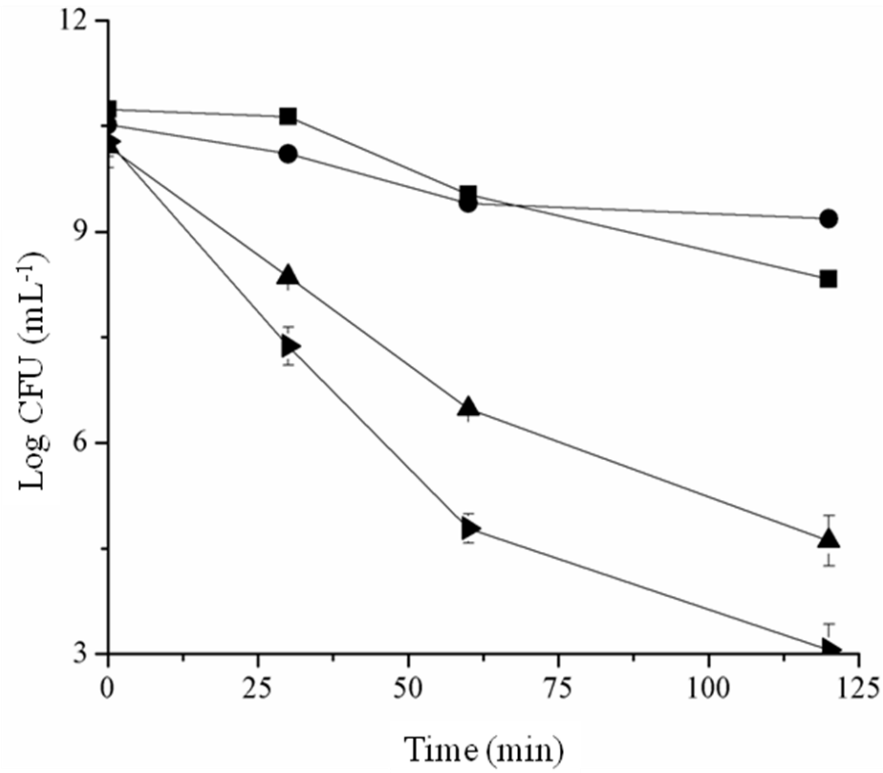

Figure 4 Cell viability of LPMP when incubated under simulated intestinal juice (SIJ) and simulated gastric juice (SGJ): (●) LPMP under SIJ; (■) LPMP under SGJ; (▲) Free cells under SIS; (►) Free cells under SGS. Results are the mean of triplicates.

Sultana *et al.*[2] immobilized *L. acidophilus* in alginate-starch complex microcapsules that were incubated under simulated high acid gastric conditions (pH 2), producing reductions of 5 log CFU g^{-1} (3.2×10^6 CFU mL^{-1}) after 3 h of incubation, from an initial cell concentration of 3.8×10^{11} CFU mL^{-1}. Mokarram *et al.*[40] showed that when calcium–alginate capsules containing *L. acidophilus* were incubated under simulated gastric juice (pH 1.5), the cell viability reduced by 3-log cycles. Coating the capsules with 1 or 2 layers of sodium alginate only improved the survival of cells by 1 and 2 log cycles, respectively.

Finally, the survival of *L. acidophilus* in gastric fluid (pH 1.2) was studied by Laelorspoen *et al.*[41] of cells encapsulated in alginate and citric acid-modified zein coating by electrospraying. After incubation of the cell systems in simulated gastric fluid (pH 1.2), the authors obtained survivals of 7.14 log CFU mL^{-1} compared to 4.52 log CFU mL^{-1} for free-cell suspensions (control) at 37 ºC for 2 h.

The results obtained in this work suggest that the microencapsulation matrix was effective in protecting cells against SGJ and SIJ, and that the technique of electrospraying was appropriate to prepare these microcapsules, being a promising alternative for the encapsulation of probiotics.

The technique of electrospraying can produce microcapsules with controlled and uniform size, without using excessive heat, promoting good results of cell viability, as demonstrated in this study. Also important is the fact that the microcapsules can support high amounts of cells, as seem in Figure 1.[20,41,42] There are many companies, which commercialize electrospray/electrospinning equipment with high loading efficiencies, thus the scaling-up of this technique is readily available. Besides, there is a full control of diameter size by varying only the flow rate and conductivity.

3.4 Microbiological quality of the prepared LPMP

The microbiological quality of foods that are not heat-treated and stored at room temperature can be a sensitive aspect in food security and of concern for food formulations. Because LPMP would be intended to be stored at 25 ºC ± 1 ºC, we decided to investigate the microbiological profile of it immediately after freeze-drying and again after six months of storage at 25 ºC. According to the results obtained (Table 1), pathogens were not found in samples (initial and after six months), concluding that the LPMP can be safely stored at room temperature.

Table 1 Microbiological parameters and results interpretations of LPMP at initial time and after six months of storage at 25 ºC.

Samples	Microbiological Parameters[28]	Results interpretations[a28]
LPMP initial	*Bacillus cereus*	A/0.75g
	Coliforms	< 0.3 NMP/0.75g
	Coliforms 45 ºC	< 0.3 NMP/0.75g
	Salmonella spp.	A/0.75g
	Staphylococcus coagulase positive	A/0.75g
LPMP after 6 months		
	Bacillus cereus	A/0.75g
	Coliforms	< 0.3 NMP/0.75g
	Coliforms 45 ºC	< 0.3 NMP/0.75g
	Salmonella spp.	A/0.75g
	Staphylococcus coagulase positive	A/0.75g

[a]A: absent;

3.5 Sensory analysis

It would be important to know whether the addition of microspheres of probiotics to food could interfere with the sensorial perception and acceptability of such food by consumers. We devised a simple sensory test, using natural orange juice (to avoid dairy products, usually associated with LAB). According to the results obtained in the panel test (Table 2), all attributes of the product containing LPMP showed no significant differences compared to natural orange juice without probiotic (control). When the 90 panelists were asked about purchase intent of orange juice, 53 % of them answered that they would buy the juice added of probiotic, 45 % would buy the juice without the addition of probiotics, and 2 % would not buy any of the juices. These results and those showed in Table 2 suggest that the LPMP added to natural orange juice had a good acceptance, without causing perceptible changes and demonstrating that LPMP could be added to daily diets without interfering with the specific sensory characteristics of the food or its preparations.

Table 2 Acceptance of the sensory attributes of the natural orange juice (control) and natural orange juice containing microencapsulated *L. plantarum* BL011.

Sensory attributes	Product	
	Natural orange juice (control)	Natural orange juice containing probiotic (10 log CFU 200 mL^{-1})
Appearance	8.2 ± 0.7^{a}	8.3 ± 0.8^{a}
Color	8.2 ± 0.7^{a}	8.2 ± 0.8^{a}
Odor	7.5 ± 1.4^{a}	7.7 ± 1.3^{a}
Taste	7.7 ± 1.3^{a}	7.8 ± 1.6^{a}
Viscosity	7.7 ± 1.6^{a}	7.6 ± 1.9^{a}
Overall acceptability	7.8 ± 1.0^{a}	8.0 ± 1.1^{a}

*Same letters in the same line are indicating values with no statistical difference

CONCLUSION

Our study showed that it is possible to obtain a potentially probiotic powder using the microencapsulation technique of electrospraying and drying of the microspheres by freeze-drying. The LPMP showed significant cell survival under conditions of gastrointestinal juices environments tested, as well as during the long storage time of six months at 25 ºC, maintaining cell viabilities within the international parameters required for probiotics added to food. The LPMP, when added to natural orange juice, demonstrated good acceptance (> 88 %) by consumers in sensory analysis, with changes in the tasting of the orange juice not being noticeable. Our results suggest that LPMP could be interesting option for the consumption of probiotic bacteria in non-dairy products and without the requirement of cold chain maintenance, easing consumption of the product by the general population.

Acknowledgements

The authors wish to thank CNPq and CAPES (Brazil) for the financial support and scholarships of this work.

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CAPÍTULO VI – CONSIDERAÇÕES FINAIS

O presente trabalho por meio da percepção da necessidade de diversificação de produtos potencialmente probióticos oferecidos à população, e da dificuldade de inserção dos produtos atuais disponíveis devido à necessidade da cadeia do frio, buscou o desenvolvimento de tecnologia de produção de microrganismo potencialmente probiótico, microencapsulado através da tecnologia de eletrospraying. Os resultados encontrados foram promissores e demonstraram a importância do uso de um resíduo na produção de biomassa, dando um destino ao efluente e gerando um produto de alto valor agregado.

A técnica de microencapsulação utilizada possibilitou a produção de microcápsulas de tamanho adequado para incorporação em alimentos, e que apresentam resistência quando expostas aos sucos gastrointestinais e aos testes de armazenamento.

A análise sensorial demonstrou boa aceitação quando o pó alimentício foi adicionado ao suco de laranja, não havendo percepção de sua adição. Além disso, o produto obtido não necessita manutenção da cadeia do frio, podendo ser armazenado a temperatura ambiente sem perda considerável de viabilidade, quando analisado no período de seis meses. Possibilitando desta forma o consumo de microrganismos potencialmente probióticos que desepenham benefícios a saúde, às pessoas mais distantes dos grandes centros urbanos especialmente no Norte e Nordeste, e regiões específicas no interior do país.

Complementando as informações acima, o meio de cultivo utilizado e o pó alimentício obtidos, podem ser uma opção alimentícia para pessoas com patologias específicas como alergia a proteína do leite, intolerância a lactose, colesterolemia, e os vegetarianos, indivíduos que por diferentes motivos não podem consumir produtos lácteos, onde na grande maioria, são encontrados os microrganismos probióticos.

Desta forma pode-se concluir que o estudo atingiu os objetivos propostos e apresenta interesse biotecnológico, abordando desde a questão ambiental com a utilização de um resíduo agro-industrial, até a elaboração de um produto alimentício com valor agregado.

PERSPECTIVAS

O presente trabalho demonstrou importante potencial biotecnológico para produção de biomassa de *L. plantarum* BL011 a partir de resíduo agro-industrial, bem como apresentou alta capacidade de sobrevivência do microrganismo quando microencapsulado e submetido à condições adversas. Obtendo-se no final da pesquisa um pó potencialmente probiótico para adição direta nos alimentos. Contudo, melhorias ainda podem ser feitas visando aprimorar ainda mais este trabalho. Dessa forma, o avanço nos estudos pode ser realizado a partir de algumas sugestões:

- Realizar testes adicionais necessários para poder afirmar com maior precisão que a linhagem em estudo é um microrganismo probiótico.
- Estudar a produção submersa através de batelada alimentada, visto que esta técnica geralmente apresenta resultados promissores em relação à produção de biomassa e ao consumo dos açúcares presentes em diversos meios de cultivo.
- Estudo de outros modelos de biorreator para a produção de biomassa, como biorreator com membranas de eletrodiálise para retirada constante do ácido láctico produzido, evitando o consumo deste metabólito.
- Realizar o cultivo em biorreator utilizando outros resíduos da indústria objetivando a produção de biomassa.
- Testar outros agentes encapsulantes de uso em alimentos.
- Estudar a viabilidade econômica do processo de produção do pó alimentício.
- Estudar o comportamento do produto obtido em outros sistemas alimentícios.

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ANEXOS

ANEXO I - Termo de consentimento livre e esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Avaliação de suco natural de laranja com adição de probióticos

Prezado (a) Senhor (a):

Representamos um grupo de pesquisadores da Universidade Federal do Rio Grande do Sul (UFRGS) e do Instituto de Ciência e Tecnologia de Alimentos (ICTA) que está realizando um estudo sobre a produção de um pó alimentício com microrganismo imobilizado. O objetivo da pesquisa é desenvolver um pó alimentício para adicionar em alimentos, como uma alternativa para aumentar o consumo de probióticos.

Pretende-se realizar análise sensorial do suco natural de laranja com adição de probiótico. Os testes de aceitação serão realizados no Laboratório de Análise Sensorial do ICTA. Os provadores receberão 2 amostras codificadas (servidos em copos plásticos brancos com diferentes codificações de 3 dígitos) referentes aos diferentes processamentos, 1 copo de água para ser consumido entre as amostras e uma ficha para avaliar as amostras quanto a aceitação dos atributos aparência, cor, odor, sabor, viscosidade e a aceitação global, utilizando escala hedônica estruturada de 9 pontos, onde 1 corresponde a "desgostei muitíssimo", 5 "nem gostei nem desgostei" e 9 a "gostei muitíssimo".

O teste não deve ser realizado por indivíduos alérgicos aos ingredientes da formulação (alginato de sódio alimentício e *Lactobacillus plantarum* BL011). O participante pode desistir de provar as amostras em qualquer uma das etapas sem sofrer qualquer prejuízo. Conforme resolução 466/12, "Considera-se que toda pesquisa envolvendo seres humanos envolve riscos, sendo estes de graduação variada. No caso, trata-se da ingestão de alimentos manipulados com todas as normas de boas práticas de fabricação o que reduz os riscos de possível ingestão de produtos não adequados. Existe risco de reações alérgicas, no caso do provador possuir alergia a algum dos componentes da formulação".

O participante não terá custos com a realização do teste, e não será concedido qualquer tipo de benefício financeiro pela participação. A avaliação deste estudo será

mantida em sigilo. As informações do participante (nome, idade, sexo) não serão divulgadas e nem passadas a outros estudos, e não será necessário informar o nome do responsável no estudo.

Em caso de qualquer dúvida, o senhor (a) poderá entrar em contato com o pesquisador responsável ou com o Comitê de Ética em Pesquisa do UFRGS pelos telefones:

Pesquisador responsável Marco Antônio Záchia Ayub
Celular: 94355151
Telefone: 3308-6685

Comitê de Ética em Pesquisa da UFRGS – Telefone 3308-4085
Porto Alegre, ____ de _____de 2014.
Eu, _____ recebi as orientações necessárias para entender o presente estudo, e desejo participar da pesquisa.

_____ _____

Assinatura do participante Assinatura do pesquisador

Obs: O presente documento, baseado no item IV das Diretrizes e Normas Regulamentadoras para a Pesquisa em Saúde, do Conselho Nacional de Saúde (Resolução 466/12) será assinado em duas vias, de igual teor, ficando uma via em poder do participante e a outra com o pesquisador responsável.

ANEXO II – Ficha sensorial

Análise sensorial de suco natural de laranja com adição de probiótico

Código:

Data:...../...../.....

Idade:.....

PROCEDIMENTOS:

Você está recebendo duas amostras de suco natural de laranja. Avalie as características de aparência, cor, odor, sabor, viscosidade e aceitação global, seguindo a escala abaixo:

Aceitação

1- desgostei muitíssimo

2- desgostei muito

3- desgostei moderadamente

4- desgostei levemente

5- nem gostei nem desgostei

6- gostei levemente

7- gostei moderadamente

8- gostei muito

9- gostei muitíssimo

Atribua a cada característica uma nota de acordo com a tabela acima. Proceder, avaliando primeiro a aparência, cor e odor. Através de degustação, avaliar sabor, viscosidade e aceitação global. Prove as amostras da esquerda para a direita, lembrando de beber água entre as amostras.

Atributos	**Amostra 371**	**Amostra 453**
Aparência		
Cor		
Odor		
Sabor		
Viscosidade		
Aceitação global		

Você compraria algum desses sucos? Qual?

_____

ANEXO III - Aprovação do projeto pelo Comitê de Ética

UFRGS UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Lactobacilos em processos biotecnológicos: aplicação em formulação alimentar
Pesquisador: Marco Antônio Záchia Ayub
Área Temática:
Versão: 2
CAAE: 34835314.7.0000.5347
Instituição Proponente: Universidade Federal do Rio Grande do Sul
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 908.972
Data da Relatoria: 26/11/2014

Apresentação do Projeto:
Esse é um projeto de pós-graduação do Programa de Pós-Graduação em Tecnologia dos Alimentos, intitulado METABOLISMO DE LACTOBACILOS EM PROCESSOS BIOTECNOLÓGICOS E APLICAÇÃO EM FORMULAÇÃO ALIMENTAR.

Objetivo da Pesquisa:
O objetivo é a produção de microrganismos probióticos em sistema de biorreator submerso definindo parâmetros de processo, tais como a formulação de meio de cultivo vegetal e regime de operação do biorreator, bem como a aplicação do probiótico em sistemas imobilizados e posteriormente adição em alimento modelo, que será testado pelos participantes da pesquisa.

Avaliação dos Riscos e Benefícios:
Não há benefícios diretos para os participantes da pesquisa. A pesquisa poderá ajudar a desenvolver novos e melhores alimentos. Os riscos para os participantes estão em provar esse novo alimento e estão agora explicitados no TCLE.

Comentários e Considerações sobre a Pesquisa:
É um projeto interessante sobre desenvolvimento de novos alimentos.

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

Considerações sobre os Termos de apresentação obrigatória:

Os autores implementaram as modificações sugeridas pelo CEP, tornando o projeto mais adequado.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Os autores resolveram as principais pendências. No entanto, foi informado que o convite para participar da pesquisa será feito de forma oral para alunos, professores e funcionários. Conforme solicitado em parecer anterior, este convite deve ser feito por cartaz (para evitar questões de constrangimento e hierarquia). Assim, solicito o modelo do cartaz a ser afixado, para avaliação do CEP.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado.

PORTO ALEGRE, 11 de Dezembro de 2014

Assinado por:
MARIA DA GRAÇA CORSO DA MOTTA
(Coordenador)