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Encapsulação de Lactobacillus acidophilus (La-05) e Bifidobacterium lactis (Bb-12) e avaliação "in vitro", do nível de tolerância dos mesmos às secreções gastrintestinais

PARECER

Este exemplar corresponde à redação final da tese defendida por Carmen Sílvia Fávaro Trindade, aprovada pela Comissão Julgadora em 11 de abril de 2001.

Campinas, 11 de abril de 2001

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ao Marco e ao Pedro

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RESUMO GERAL

Esse trabalho visou a encapsulação de *Lactobacillus acidophilus* e *Bifidobacterium lactis* e a avaliação "in vitro" da tolerância desses microrganismos livres e encapsulados quando inoculados em soluções biliares e de pHs ácidos, ou seja, em condições semelhantes às encontradas no intestino e no estômago humano, respectivamente. Além disso, foi determinada a estabilidade de *B. lactis* livres e encapsulados, incorporados em iogurte que foi mantido sob refrigeração, durante 28 dias. Para tanto, foram utilizados como agentes encapsulantes os polímeros entéricos aceto fitalato de celulose e alginato e os métodos de microencapsulação por *spray drying* e de imobilização por extrusão, respectivamente. *Lactobacillus acidophilus* e *Bifidobacterium lactis* foram extremamente resistentes às soluções biliares, tanto livres, quanto encapsulados. Apresentaram boa resistência quando inoculados em soluções de pH 2. Entretanto, nas soluções de pH 1, as populações das células livres e imobilizadas em alginato foram dizimadas, enquanto as células microencapsuladas em aceto fitalato de celulose apresentaram uma maior resistência. *B. lactis* não apresentaram uma boa viabilidade em iogurte, em nenhuma das condições testadas. Foi possível encapsular *Lactobacillus acidophilus* e *Bifidobacterium lactis* pelos métodos de extrusão e por *spray drying* mantendo um número elevado de células viáveis. A imobilização em alginato de sódio não foi eficaz na proteção às células de *Lactobacillus acidophilus* e *Bifidobacterium lactis* e a microencapsulação em aceto fitalato de celulose foi efetiva na proteção dos mesmos inoculados em soluções ácidas, embora não tenha sido eficiente quando *B. lactis* microencapsulado foi inoculado em iogurte.

GENERAL SUMMARY

This study aimed at encapsulating *Lactobacillus acidophilus* and *Bifidobacterium lactis* and at carrying out an "in vitro" study of the tolerance of these microorganisms, both free and encapsulated, when inoculated into bile solutions and acid pH solutions, that is, into conditions similar to those encountered in the human intestine and stomach respectively. In addition, the stability of free and encapsulated *B. lactis* was determined, when inoculated into yoghurt and stored under refrigeration for 28 days. The encapsulating agents used for this purpose were the enteric polymers cellulose acetate phthalate and alginate, and the methods of microencapsulation were spray drying and immobilisation by extrusion, respectively. *Lactobacillus acidophilus* and *Bifidobacterium lactis* were extremely resistant to bile solutions, both in the free forms and encapsulated. They also presented good resistance when inoculated into solutions at pH 2.0. However at pH 1.0, both the free cells and those encapsulated in alginate were destroyed, whilst those encapsulated in cellulose acetate phthalate showed greater resistance. *B. lactis* showed poor viability in yoghurt, under all conditions tested. It was possible to encapsulate *Lactobacillus acidophilus* and *Bifidobacterium lactis* by both the methods of extrusion and by spray drying, retaining an elevated number of viable cells. Immobilisation in sodium alginate was not efficient in protecting cells of *Lactobacillus acidophilus* and *Bifidobacterium lactis,* and microencapsulation was efficient in protecting these same organisms when inoculated into acid solutions, although it was not efficient when microencapsulated *B. lactis* was incorporated into yoghurt.

INTRODUÇÃO GERAL

O consumo de leites fermentados remonta à história escrita. Entretanto, desde o início de 1907, quando o microbiologista russo Metchnikoff propôs uma teoria na qual o consumo de grandes quantidades de leites fermentados prolongaria a vida, muitos pesquisadores têm se empenhado no estudo dos microrganismos empregados na elaboração de produtos lácteos e no efeito destes sobre os homens e animais. Assim, recentemente surgiu o conceito de probiótico, o qual pode ser definido como microrganismos que causam efeitos benéficos nos indivíduos que os ingerem.

Os probióticos emergiram como um dos ingredientes funcionais que mais influenciam a fisiologia e a função gastrintestinal, por apresentarem propriedades antimicrobianas contra uma grande variedade de patógenos, anticarcinogênica e anticolesterolêmica, entre outras.

Lactobacillus acidophilus, L. casei e bifidobactérias são exemplos de microrganismos probióticos. Entretanto, para que exerçam alguma atividade benéfica ao consumidor, esses microrganismos precisam atender a uma série de requisitos, como estar viáveis no alimento ($\geq 10^6$ ufc/ml) no momento da ingestão e sobreviver à passagem pelo sistema gastrintestinal.

A incorporação desses microrganismos em alimentos ainda é limitada pelo fato de que estes normalmente não contribuem beneficamente para a qualidade sensorial do produto. Também há dúvidas a respeito da sua sobrevivência nos alimentos, uma vez que ainda não existem métodos certificados para a contagem e identificação, além da extrema sensibilidade desses microrganismos a uma série de fatores como presença de oxigênio, outras culturas e pH baixo.

A microencapsulação é uma tecnologia relativamente nova que tem solucionado dificuldades no emprego de ingredientes alimentícios, uma vez que pode reduzir a reatividade e aumentar a estabilidade destes em condições ambientais adversas, como na presença de luz, oxigênio e pHs extremos. Logo, essa técnica pode ser uma alternativa eficiente para a proteção de

microrganismos probióticos e que possibilitaria uma alta taxa de sobrevivência durante a vida útil do alimento e durante a passagem pelo sistema gastrintestinal.

Assim, os objetivos desse trabalho foram:

- imobilizar *B. lactis* e *L. acidophilus* em alginato de cálcio, pelo método de extrusão e estudar a tolerância desses, na forma livre e imobilizada, em níveis de pH e bile semelhantes aos encontrados no estômago e intestino humano, respectivamente.
- avaliar a estabilidade de *B. lactis* e *L. acidophilus* livres e imobilizados em alginato de cálcio, em leite acidificado; determinar a estabilidade de *B. lactis* imobilizados em iogurte e verificar a eficiência de dois meios de cultura, um seletivo e outro diferencial, para contagem de *B. lactis* em iogurte.
- elaborar microcápsulas com propriedades simbióticas utilizando para tanto os microrganismos probióticos *L. acidophilus* e *B. lactis*, um fruto-oligossacarídeo como prebiótico, aceto fitalato de celulose como material de parede e o método de microencapsulação por "spray drying"; verificar o efeito de diferentes temperaturas de secagem sobre os microrganismos; caracterizar as microcápsulas microscopicamente e através da distribuição e do tamanho das partículas e determinar a estabilidade dos microrganismos microencapsulados durante 90 dias de estocagem.
- avaliar a tolerância de *Bifidobacterium lactis* e *Lactobacillus acidophilus* microencapsulados em aceto fitalato de celulose, por "spray drying", em níveis de pH e bile similares aos encontrados no estômago e intestino humano, respectivamente, e determinar a sobrevivência de *B. lactis* livres e microencapsulados adicionados em iogurte.

MICROENCAPSULAÇÃO DE MICRORGANISMOS

1. Resumo

A microencapsulação é uma tecnologia relativamente nova que tem sido empregada com êxito na indústria de cosméticos, farmacêutica e alimentícia. Essa técnica tem solucionado limitações no emprego de ingredientes e aditivos alimentícios, uma vez que pode suprimir "flavors" indesejáveis, reduzir a volatilidade e a reatividade e aumentar a estabilidade destes em condições ambientais adversas, como na presença de luz, oxigênio e pHs extremos. Embora a encapsulação de microrganismos tenha sido pouco explorada, pode ser uma alternativa eficaz para proteger probióticos incorporados em alimentos ácidos, com grande quantidade de oxigênio dissolvido, elaborados com outras culturas e durante a passagem pelo sistema gastrintestinal.

2. Summary

Microencapsulation is a relatively new technology which has been used with considerable success in the cosmetic, pharmaceutical and food industries. This technique has resolved limitations in the use of food ingredients and additives, since it can overcome undesirable flavours, reduce volatility and reactivity and increase their stability under adverse environmental conditions such as the presence of light, oxygen and extreme pH values. Although the encapsulation of microorganisms has been little explored, it could be an efficient alternative to protect probiotics incorporated into acid foods, those containing large amounts of dissolved oxygen, and those prepared together with other cultures, as well as during their passage through the gastrointestinal tract.

3. Introdução

Microencapsulação é a tecnologia de empacotamento com finas coberturas poliméricas aplicáveis em sólidos, gotículas de líquidos ou material gasoso, formando pequenas partículas denominadas microcápsulas, que podem liberar seu conteúdo sob velocidade e condições específicas (TODD, 1970).

Embora a microencapsulação venha sendo estudada desde 1930, sua primeira aplicação comercial ocorreu por volta de 1950, por Green e Scheicher, para a produção de papel cópia sem carbono (ARSHADY, 1990).

Microcápsulas podem ser descritas como embalagens extremamente pequenas, compostas por um polímero como material de parede e um material ativo chamado de núcleo. O ingrediente ativo pode ser um aditivo alimentício, um medicamento, um adesivo ou outros materiais (ARSHADY, 1993).

Quando comparada com as embalagens convencionais, as quais normalmente são empregadas para facilitar transporte, armazenagem, manipulação e apresentação, as microcápsulas são geralmente empregadas para melhorar a performance do material ou criar novas aplicações (ARSHADY, 1993). Portanto, os propósitos gerais da microencapsulação podem ser: fazer um líquido comportar-se como sólido, separar materiais reativos, reduzir toxidez do material ativo, controlar liberação do material, reduzir volatilidade ou flamabilidade de líquidos, mascarar gosto de componentes amargos, aumentar "shelf-life", proteger contra luz, água, calor e outros (JACKSON & LEE, 1991).

Entre os materiais que podem ser encapsulados, para aplicação na indústria alimentícia, incluem-se ácidos, bases, óleos, vitaminas, sais, gases, aminoácidos, flavors, corantes, enzimas e microrganismos (JACKSON & LEE, 1991).

4. Agentes encapsulantes e mecanismos de liberação

Muitos materiais podem ser utilizados como cobertura para as microcápsulas entre eles as gomas arábica, agar, alginato e carragena; os carboidratos amido, dextrinas e sacarose; as celuloses carboximetilcelulose, acetilcelulose, nitrocelulose; os lipídeos parafina, mono e diglicerídeos, óleos e gorduras; os materiais inorgânicos sulfato de cálcio e silicatos; as proteínas glúten, caseína, gelatina e albumina (JACKSON & LEE, 1991).

O tamanho das microcápsulas pode variar de alguns micrômetros até vários milímetros; a forma também é bastante variável em função do método e agente encapsulante utilizado para prepará-las (JACKSON & LEE, 1991).

A escolha do agente encapsulante depende de uma série de fatores, entre eles a não reatividade com o material a ser encapsulado, o processo utilizado para a formação da microcápsula, o mecanismo de liberação ideal etc. Normalmente, polímeros hidrossolúveis são utilizados para microencapsular núcleos lipossolúveis e vice-versa (BAKAN, 1973).

As características de liberação do material ativo microencapsulado variam de acordo com a natureza do agente encapsulante, sendo que normalmente ocorrem devido a mecanismos como: variação de temperatura e de pH, solubilidade do meio, biodegradação, por difusão, ruptura mecânica, permeabilidade seletiva, gradiente de concentração existente em relação a cobertura etc (BAKAN, 1973; BRANNON-PEPPAS, 1993). É importante ressaltar que a espessura da cobertura da microcápsula pode ser manipulada de forma que a estabilidade e a permeabilidade sejam alteradas (BAKAN, 1973).

Alguns materiais utilizados como agentes encapsulantes e os prováveis mecanismos de liberação das microcápsulas são apresentados no Quadro 1.

Quadro 1 - Agentes encapsulantes e seus prováveis mecanismos de liberação.

Agentes encapsulantes	Mecanismos de liberação			
	Mecânico	Térmico	Dissolução	Químico
Agentes encapsulantes hidrossolúveis				
Alginato	•		•	
Carragena	•		•	
Caseínato	•		•	
Celulose derivatizada	•		•	
Quitosana	•			
Gelatina	•		•	
Goma xantana	•	•		
Goma arábica	•	•		
Látex	•		•	
Polietileno-glicol	•	•	•	
Óxido de polietileno	•	•	•	

Polipectato	•		•	
Polivinil-álcool	•		•	
Amido	•		•	
Açúcar derivatizado	•	•	•	•
Agentes encapsulantes insolúveis em água				
Etilcelulose	•			
Polímero de etileno-vinil acetato	•	•		
Álcoois-graxos	•	•		•
Ácidos-graxos	•	•		•
Resinas de hidrocarbonetos	•	•		
Hidroxipropil metilcelulose fitalato	•			•
Mono-diglicerídeos, triglicerídeos	•	•		
Parafina, ceras naturais	•	•		
Polianidridos	•		•	•
Polibuteno	•	•		
"Polylactide"	•		•	•
"Polylactide-co-glycolide"	•		•	•
Polietileno	•	•		
Ésteres de polimetacrilato	•		•	•
Poliortoésteres	•		•	•
Fitalato de polivinil-acetato	•			•
Saran®	•			
Shellac	•			•
Cloreto de vinilideno/acrilonitrilo	•			
Zeína	•			

Fonte: SOUTHWEST RESEARCH INSTITUTE, 1991.

4.1. Alginatos

Alginatos são polímeros lineares de alto peso molecular, extraídos de algas. Nessa classe de materiais incluem-se uma grande variedade de

compostos constituídos de unidades estruturais dos ácidos D-Manurônico (M) e L-Gulurônico (G), os quais são arranjados em regiões compostas apenas por uma unidade, por exemplo blocos-M (MMMMM) ou por ambas, ou seja, blocos onde se altera G e M (por exemplo MGMGMG). Na cadeia do polímero, os monômeros tendem a encontrar sua posição energeticamente mais favorável dando origem à estrutura do gel (SHAHIDI & HAN, 1993; ONSOYEN *apud* IMENSON, 1997). Sob condições ácidas o alginato ou, mais corretamente, o ácido algínico é insolúvel. Sob condições neutras ou básicas, os grupamentos estão ionizados o que torna o alginato hidrossolúvel. Assim, o alginato pode ser considerado um polímero entérico que é insolúvel em pH ácido, mas solúvel em condições de pH neutro e básico, quando este forma um sal de sódio, potássio, amônio ou magnésio (THIES, 1994).

Segundo ONSOYEN *apud* IMENSON (1997), a característica crucial, que propícia as múltiplas aplicações do alginato na indústria alimentícia, é sua capacidade de formar gel.

Para explicar o mecânismo de formação do gel de alginato foi proposto um modelo denominado "caixa de ovos". De acordo com este modelo, as cadeias de blocos G alinhadas lado a lado são interligadas por íons Ca+, dando origem a uma rede tridimensional (THIES, 1994).

Géis de alginato são termo-resistentes e estaveis ao congelamento e descongelamento (ONSOYEN *apud* IMENSON, 1997). Porém, estes géis são instaveis à presença de íons fosfatos e lactatos, o que os torna pouco resistentes ao crescimento de bactérias ácido láticas devido a possíveis modificações químicas que ocorreriam nos mesmos (AUDET *et al.*, 1989).

4.2. Aceto fitalato de celulose

Aceto fitalato de celulose é um produto da reação de grupamentos fitalatos com um éster de aceto de celulose (USP, 1985). Devido à presença de grupamentos ionizáveis (grupo fitalato) esse polímero é insolúvel em meio ácido pH $\leq$ 5, mas é solúvel quando o pH é $\geq$ 6. Em adição, aceto fitalato de celulose é fisiológicamente inerte quando ministrada "in vivo" e é, por essa razão, largamente utilizada como agente encapsulante para liberação de drogas e outras substâncias farmacêuticas no intestino (RAO *et al.*, 1989).

Segundo SVOBODA *et al.* (1993), soluções de aceto fitalato de celulose não tamponadas são incapazes de proteger agentes sensíveis ao pH ácido pelo tempo requerido para alcançar o intestino.

Plasticizante é definido como uma substância com alto ponto de fusão, que quando adicionado em outro material provoca mudanças nas propriedades físicas, químicas e mecâncias do mesmo, com incremento de flexibilidade e resistência mecânica e a redução de possíves zonas descontínuas e quebradiças (FENNEMA, 1994). Por suas características químicas, pode influenciar fortemente as propriedades funcionais dos filmes e suportes poliméricos. RAFFIN *et al.* (1996) reportaram que a presença de plasticizante provoca decréscimo na permeabilidade de filmes de aceto fitalato de celulose a íons H+.

BÉCHARD *et al.* (1995) obtiveram uma retenção de 99% de ácido acetilsalicílico encapsulado em filme de aceto fitalato de celulose, através da técnica de *pan coating*, após 2 horas de inoculação em solução de HCl 0,1 N e em torno de 5% após 1 hora em solução tampão fosfato pH 6,8. Isto comprova a eficácia do polímero para aplicações entéricas.

Invertase microencapsulada em aceto fitalato de celulose, pelo método da emulsificação dupla, reteve 40% da atividade inicial depois de 1 hora de incubação em suco gástrico, enquanto a atividade da enzima livre foi extremamente afetada pelo pH baixo (GARCIA *et al.*, 1989).

TAKENAKA *et al.* (1980) elaboraram microcápsulas da droga sulfametoxazol com ação entérica eficiente, utilizando para tanto, aceto fitalato de celulose como material encapsulante e a técnica "spray drying".

5. Métodos utilizados para microencapsulação

Existem várias técnicas que podem ser utilizadas para microencapsulação de ingredientes alimentícios, sendo que a seleção do método é dependente da aplicação que será dada à microcápsula, tamanho desejado, mecanismo de liberação e propriedades físico-químicas tanto do material ativo, quanto do agente encapsulante (JACKSON & LEE, 1991). Alguns métodos utilizados para encapsulação e as respectivas faixas de tamanho das microcápsulas são apresentadas no Quadro 2.

Quadro 2 - Métodos utilizados para encapsulação e tamanhos das cápsulas.

Métodos de encapsulação	Materiais encapsuláveis	Faixa de tamanho (μm)
Métodos físicos		
Extrusão estacionária	Líquido/Sólido/Gás	1.000 – 6.000
Bocal submerso	Líquido/Sólido/Gás	700 – 6.000
Extrusão centrífuga	Líquido/Sólido/Gás	125 – 3.000
Bocal vibrante	Líquido/Sólido/Gás	500 – 2.000
"Spray drying"	Líquido/Sólido	5 – 150
Disco rotativo	Líquido/Sólido	5 - 1.000
"Pan coating"	Sólido	>500
Suspensão por ar	Sólido	50 – 10.000
Métodos químicos		
Coacervação	Líquido/Sólido	1 – 500
Polimerização interfacial	Líquido/Sólido	1 – 500
Evaporação do solvente	Líquido/Sólido	1 - 5.000
Polimerização "in situ"	Líquido/Sólido	1 – 500

Fonte: SOUTHWEST RESEARCH INSTITUTE, 1991.

Além dos métodos citados no Quadro 2, ARSHADY (1993) ainda faz referência à utilização de "spray freezing", "spray chilling" e "spray cooling", como métodos efetivos para microencapsulação.

5.1. Microencapsulação pelo processo "spray drying"

A produção de microcápsulas via secagem por atomização, ou seja, pelo método "spray drying", é a mais utilizada na indústria alimentícia (JACKSON & LEE, 1991). Segundo BRANNON-PEPPAS (1993), esse processo oferece as vantagens de ser de baixo custo, ter grande diversidade e disponibilidade de equipamentos e permitir a utilização de uma grande variedade de materiais de parede. Porém, a dispersibilidade das cápsulas pode ser difícil devido à formação de partículas extremamente pequenas.

Segundo RÉ (1998), esta técnica de microencapsulação é aplicável para materiais ativos termo-sensíveis devido à rápida evaporação do solvente que

compõe as gotículas. Para JACKSON & LEE (1991), esse processo pode ser utilizado para esses materiais desde que a secagem seja muito rápida e o material de cobertura seja aquecido a temperaturas menores que 100°C, porém, perdas podem ocorrer.

Este processo envolve basicamente as seguintes etapas: formação de uma emulsão ou a suspensão do agente encapsulante e do material ativo, atomização da emulsão em uma câmara de secagem contendo ar quente circulando e secagem das gotículas atomizadas (JACKSON & LEE, 1991; SHAHIDI & HAN, 1993).

O agente encapsulante ideal para esse processo deve possuir boa capacidade emulsificante e de formação de filme, ter baixa viscosidade em altas concentrações de sólidos (500 cps ao nível $\geq$ 45% de sólidos), exibir alta higroscopicidade, sabor pouco acentuado, estabilidade e boa proteção ao material ativo. Hidrocolóides como gelatina, gomas vegetais, amidos, dextrina e proteínas não geleificantes geralmente são utilizados como agentes encapsulantes nesse processo (SHAHIDI & HAN, 1993; JACKSON & LEE, 1991).

Microcápsulas obtidas por atomização que apresentam fissuras na superfície podem ter sua eficiência comprometida. A microscopia eletrônica de varredura fornece informações sobre a morfologia das microcápsulas além de permitir a avaliação da integridade das mesmas (ROSENBERG *et al.*, 1985).

5.2. Microencapsulação por extrusão

A encapsulação de ingredientes alimentícios por extrusão é um processo relativamente novo quando comparado ao "spray drying". A palavra extrusão aqui tem um sentido diferente daquele utilizado para cozimento e texturização de produtos a base de cereais (SHAHIDI & HAN, 1993).

O método mais comumente utilizado para encapsulação/imobilização de microrganismos é o da extrusão de uma suspensão de células/polímero (alginato) através de uma seringa usando ar ou força mecânica. As gotas caem em uma solução salina e geleificam instantaneamente, assim, as células ficam retidas em uma rede tridimensional de alginato de cálcio. Os diâmetros das

cápsulas podem variar de 2 a 4 mm e normalmente a morfologia é irregular devido ao formato de gota (AUDET *et al.*, 1989).

A encapsulação de materiais ativos sensíveis em géis de polímeros naturais é uma técnica relativamente branda, porque não implica na utilização de solventes orgânicos e de altas temperaturas, entretanto, o gel tem uma vida de prateleira limitada (THIES, 1994).

O envolvimento de células microbianas em alginato de cálcio é um método de imobilização simples e de baixo custo. Além disso, o alginato é atóxico, o que possibilita a sua utilização em alimentos. A liberação do núcleo ocorre com a solubilização desse gel por seqüestrantes de íons cálcio (SHEU & MARSHALL, 1993). Esse método tem sido o mais utilizado para microencapsulação de microrganismos.

6. Microencapsulação e imobilização de microrganismos

Segundo SHAHIDI & HAN (1993) e JACKSON & LEE (1991), notavelmente existem poucos exemplos de encapsulação de microrganismos em comparação a outros ingredientes, para aplicações na área de alimentos.

Microrganismos têm sido microencapsulados ou imobilizados para possibilitar a reutilização dos mesmos na produção de ácido lático e produtos lácteos fermentados (TIPAYANG & KOZAKI, 1982; HYNDMAN *et al.*, 1993; GROBOILLOT *et al.*, 1993), para aumentar a concentração de células em reatores, aumentando a produtividade (YOO *et al.*, 1996), para protegê-los contra presença de oxigênio (KIM & OLSON, 1989), contra as baixas temperaturas de congelamento (SHEU & MARSHALL, 1993; SHEU *et al.*, 1993), contra o efeito bactericida do suco gástrico e outros meios ácidos (RAO *et al.*, 1989; MODLER & VILLA-GARCIA, 1993; DINAKAR & MISTRY, 1994; KHALIL & MANSOUR, 1998), para retirá-los do produto, interrompendo a acidificação (CHAMPAGNE & CÔTE, 1987), para aumentar a estabilidade da cultura durante a estocagem refrigerada (KIM *et al.*, 1988; CHAMPAGNE *et al.*, 1994) e para aumentar a vida útil de *Pseudomonas fluorescens-putida* (AMIET-CHARPENTIER *et al.*, 1998).

GROBOILLOT *et al.* (1993) obtiveram êxito na microencapsulação de *Lactococcus lactis* subsp *cremoris* através de ligações cruzadas utilizando

quitosana. Apesar de ter havido alguma perda de atividade da cultura durante a microencapsulação, essa foi intensificada após sucessivas utilizações. A membrana de quitosana não teve efeito inibitório no desenvolvimento da cultura.

HYNDMAN *et al.* (1993) utilizaram ligações cruzadas de gelatina para microencapsular *Lactococcus lactis* subsp *lactis*. Após a imobilização, a atividade e a viabilidade das células aumentaram devido ao crescimento que ocorreu dentro da microcápsula. Dos óleos testados para a fase de dispersão, durante o processo de imobilização, o de silicone apresentou a melhor performance.

CHAMPAGNE & CÔTE (1987) imobilizaram bactérias láticas em alginato para incorporação em creme de leite. O pH 5,5 foi obtido apenas com 2 horas de fermentação, enquanto na condição clássica levava 4 horas. Após a remoção das células imobilizadas, o creme continha 6,1 x 10^6 bactérias láticas por ml; ou seja, quase 400 vezes menos do que no método clássico. Creme fermentado com as bactérias imobilizadas foi menos propenso à acidificação durante estocagem devido à redução do número de bactérias láticas presentes, assim, a dessoragem foi retardada e ainda manteve-se o efeito inibitório dessas bactérias em virtude da presença do ácido.

A imobilização de *Lactobacillus bulgaricus* em alginato de cálcio teve um rendimento de 90% e a velocidade de adição da solução de cloreto de cálcio foi determinante para o formato das cápsulas. A cápsula de alginato ofereceu uma boa proteção aos microrganismos durante sua estocagem em freezer, em sorvete e em sobremesa congelada (SHEU & MARSHALL, 1993; SHEU *et al.*, 1993). A microencapsulação de *Lactobacillus casei* com alginato de cálcio foi otimizada através da utilização de bário e quitosana. Este método propiciou uma alta densidade celular e boa produtividade de ácido lático (YOO *et al.*, 1996). CHANG *et al.* (1996) também promoveram a microencapsulação em cápsulas de alginato de cálcio. Foi determinada uma maior atividade da invertase pelas células de *Saccharomyces cerevisiae* imobilizadas, em relação às livres; esse efeito foi atribuído à facilidade de crescimento que a microcápsula propicia devido à baixa demanda de oxigênio no seu interior.

Brevibacterium linens e metionina foram microencapsulados em gordura de leite por KIM & OLSON (1989). Essa bactéria (microencapsulada) utilizou a

metionina para produzir metanotiol e outros componentes sulfurados, contribuindo para a formação de flavor em queijo cheddar e queijos "low-fat".

A encapsulação de *Bifidobacterium longum* em gordura do leite antes da incorporação em iogurte não melhorou a sobrevivência desse microrganismo. A microcápsula não foi barreira efetiva contra condições adversas como pH baixo, meio aeróbio e a presença de outras bactérias ácido láticas (MODLER & GARCIA, 1993). No entanto, segundo DINAKAR & MISTRY (1994), a microencapsulação de *Bifidobacterium bifidum* em κ-carragena teria oferecido proteção às células, que permaneceram viáveis em queijo cheedar após 24 semanas de maturação, apesar da distribuição das microcápsulas na massa do queijo não ter sido uniforme.

Bifidobacterium pseudolongum microencapsulados através do processo de coacervação com separação de fases, utilizando o agente entérico aceto fitalato de celulose como agente encapsulante, sobreviveram melhor em condições similares às do suco gástrico do que as células livres; as perdas durante a passagem pelo suco gástrico ainda foram significativamente diminuídas com a adição de uma segunda cobertura, de cera de abelha, à microcápsula (RAO *et al.*, 1989).

KIM *et al.* (1988) aumentaram a estabilidade de culturas láticas microencapsulando-as em alginato de sódio ou carboximetilcelulose.

Bifidobacterias encapsuladas em alginato foram mais resistentes ao pH ácido da maionese do que as células livres (KHALIL & MANSOUR, 1998).

A microencapsulação, por atomização, de *Pseudomonas fluorescens-putida,* em um copolímero denominado Eudragit®, possibilitou a sobrevivência das mesmas durante um ano. Foi determinada uma relação entre sobrevivência e umidade residual, sendo que a melhor taxa de sobrevivência foi obtida qundo a umidade residual do pó estava em torno de 25% (AMIET-CHARPENTIER *et al.*, 1998).

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MICRORGANISMOS PROBIÓTICOS

1. Resumo

Culturas probióticas como bifidobactérias e *Lactobacillus acidophilus*, têm sido intensivamente estudadas e têm demonstrado características únicas como: controle do pH do intestino, através da liberação de ácidos que restringem o crescimento de patógenos e bactérias putrefativas, alívio à intolerância à lactose e propriedades anticarcinogênicas e anticolesterolêmicas. Alimentos ditos "probióticos" como sorvetes, alimentos formulados infantis, sobremesas geladas, cookies, queijos e principalmente leites fermentados, já estão à disposição dos consumidores de países como Japão, Alemanha, Canadá, Itália, Polônia, Checoslováquia, Inglaterra, Estados Unidos, Austrália e Brasil. Porém, apesar da veemente comercialização desses produtos, a sobrevivência dessas culturas deixa dúvidas e gera muitas controvérsias, uma vez que algumas cepas desses microrganismos são extremamente sensíveis a uma série de fatores como acidez, presença de oxigênio e outras culturas. Um outro problema é a dificuldade e ausência de padronização de métodos para identificação e contagem dessas culturas.

2. Summary

Probiotic cultures such as bifidobacterias and *Lactobacillus acidophilus* have been intensively studied and have demonstrated unique characteristics in the control of the intestinal pH value, by the liberation of acids which restrict the growth of pathogens and putrefactive bacteria, alleviate lactose intolerance and impart anti-carcinogenic and anti -cholesterolemic properties. Foods labelled as "probiotic", such as ice creams, formulated baby foods, refrigerated desserts, cookies, cheeses and principally fermented milks, are already available for consumption in countries such as Japan, Germany, Canada, Italy, Poland, Tchecoslovakia, England, United States of America, Australia and Brazil. However despite the vehement commercialisation of these products, the survival of these cultures is not certain and gives rise to controversies, since some of the strains of these microorganisms are extremely sensitive to a number of factors such as acidity, presence of oxygen and other cultures. Another problem is the difficulty and absence of standardised methods for the identification and counting of these cultures.

3. Introdução

FULLER (1989) definiu probiótico como "suplemento alimentar composto de células microbianas vivas, as quais tem efeitos benéficos para o hospedeiro, por melhorar o equilibrio microbiano no intestino". Comumente *Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium bifidum*, *B. longum* e *Saccharomyces boulardii* tem sido usados como probióticos em produtos para humanos (PLAYNE, 1994), porém outras espécies também são reconhecidas como probióticos.

Para exercerem efeitos probióticos essas bactérias devem ser capazes de aderir à superfície da mucosa intestinal. Os mecanismos empregados na adesão em muitos casos não estão bem definidos, mas sabe-se que estão ligados à produção de proteínas, glicoproteínas e carboidratos especificamente para esse fim ou devido a componentes da própria membrana celular que ajudariam na aderência. Entretanto, bactérias em trânsito pelo intestino também podem exercer efeitos positivos sem a aderência propriamente dita, é o caso das bactérias do iogurte, por exemplo, que hidrolisam a lactose presente no produto lácteo e não aderem ao intestino (NAIDU *et al.*, 1999).

As bifidobactérias são microrganismos que têm como habitat natural o intestino dos homens e animais de sangue quente. Elas foram isoladas e descritas pela primeira vez por Tissie, no período de 1899-1909 (NORRIS *et al. apud* MARTIN & CHOU, 1992). Desde então as mesmas tem sofrido inúmeras alterações na sua classificação e nomenclatura e, recentemente, foram reclassificadas em 24 espécies, incluindo 9 espécies encontradas nas fezes de humanos *B. bifidum, B. longum, B. infantis, B. breve, B. adolescentis, B. angulatum, B. catenulatum, B. pseudocatenulatum e B. dentium* (MITSUOKA *apud* MARTIN & CHOU, 1992).

Todas as espécies de bifidobactérias são não-esporuladas, imóveis, anaeróbias (algumas cepas são tolerantes à presença de O_2), bastonetes Gram-positivas de forma variável (eventualmente aparecem na forma de Y), catalase negativos, não produzem H_2O_2 e bacteriocinas. A utilização de açúcares varia

entre as espécies, sendo que *B. bifidum* fermentam apenas 4 carboidratos, enquanto *B. adolescentis* podem fermentar 19. Ácido acético e lático L(+) são os principais produtos da fermentação, mas também produzem ácido fórmico e succínico, acetaldeído, acetona, acetoína, diacetil e traços de etanol. Produzem as vitaminas tiamina, riboflavina e outras do complexo B e a K. Não produzem CO_2, ácido butírico e propiônico. Podem crescer numa faixa de pH de 4,5 a 8,5, sendo que a faixa ótima é de 6,5 a 7. Crescem em temperaturas que podem variar de 25 a 45°C, sendo que a faixa ótima, para as espécies de origem humana, é de 36 a 38°C, enquanto para as de origem animal é de 41 a 43°C (KURMANN *apud* MODLER *et al.*, 1990; LAROIA & MARTIN, 1990).

Carbonato ou bicarbonato (ou gás carbônico) podem facilmente ser utilizados como fonte de carbono. Ácidos orgânicos e aminoácidos não são utilizados efetivamente como fonte de carbono. Cistina ou cisteína são fontes essenciais de nitrogênio (HASSINEN *apud* MODLER *et al.*, 1990).

Os *Lactobacillus acidophilus* são encontrados no trato intestinal de humanos e animais. Apresentam-se como bastonetes com extremidades arredondadas; isolados ou em cadeias curtas e sem grânulos metacromáticos (DELLAGLIO *et al.*, 1992). São Gram-positivos, imóveis, geralmente tem dimensões de 0,6 a 0,9 x 1,5 a 6 μm, são homofermentativos e produzem ácido lático DL pela fermentação da lactose (ANÔNIMO, 1995). Algumas cepas produzem H_2O_2 e bacteriocinas (NAIDU *et al.*, 1999).

Existem um grande número de substâncias capazes de melhorar ou promover o crescimento das bactérias probióticas (KAILASAPATHY & RYBKA, 1997). Recentemente foi criado o termo prebiótico que define-se como ingredientes alimentícios não digeríveis que tem efeito benéfico ao consumidor por selecionar, estimular o crescimento e ativar o metabolismo de bactérias probióticas no intestino (GIBSON & ROBERFROID, 1995). Rafinose, estaquiose, fructo-, isomalto- e galacto-oligossacarídeos são efetivos para proliferação e implantação das bifidoctérias (MITSUOKA *apud* KAILASAPATHY & RYBKA, 1997).

Produtos simbióticos contém uma mistura de prebióticos e probióticos, os quais, em sinergia, vão beneficiar o hospedeiro (FULLER & GIBSON, 1997).

4. Atividades terapêuticas

As bactérias probióticas apresentam reconhecidamente atividade antimicrobiana contra microrganismos como *Salmonella typhimurium*, *Clostridium difficile*, *Campylobacter jejuni, Escherichia coli* e *Shigella* spp. (O'SULLIVAN *et al.*, 1992). As atividades antimicrobianas das bifidobactérias e dos lactobacilos diferem em vários aspectos: lactobacilos atuam através da produção de ácido lático, peróxido de hidrogênio e bacteriocinas; bifidobactérias não produzem peróxido, nem bacteriocinas, mas produzem ácido lático e acético. A produção desses ácidos provoca a redução do pH intestinal, o que restringe ou inibe o crescimento de muitos potenciais patógenos e bactérias putrefativas. Ainda, controlando o pH é possível restringir a produção de fenóis, amônia, esteróides, toxinas bacterianas, assim como aminas vasoconstritoras como histaminas, tiramina, cadaverina e agmatina (MODLER *et al.*, 1990b).

Bifidobactérias evitam a produção de amônia inibindo o crescimento de organismos produtores de uréia. Esse efeito pode ser considerado uma vantagem no controle sistêmico de encefalopatias (MODLER *et al.*, 1990b).

A intolerância à lactose é causada pela falta ou atividade insuficiente da enzima lactase no intestino humano. A ausência dessa enzima faz com que o indivíduo sofra uma série de desconfortos abdominais. *L. acidophilus* e bifidobactérias produzem β-galactosidase, a qual hidrolisa a lactose, melhorando a intolerância a esse açúcar (KAILASAPATHY & RYBKA, 1997).

Em tratamentos sistêmicos contra AIDS, tem sido proposta a administração via oral de lactobacilos. Estudos sugeriram que o peróxido de hidrogênio, produzido por alguns desses microrganismos, atuaria isoladamente ou em conjunto com uma peroxidase (de origem leucócita ou uterina) para inativar o vírus HIV (O'SULLIVAN *et al.*, 1992).

KAILASAPATHY & RYBKA (1997) reportaram estudos feitos com animais que confirmaram que leites fermentados com bactérias probióticas inibem a formação e a proliferação de tumores. Para explicação desse efeito foram propostos alguns mecanismos de ação, tais como a redução das enzimas produzidas por bactérias fecais, as quais catalisam a conversão de aminas pró-carcinogênicas em carcinogênicas no cólon; estimulação do sistema imunológico; ocorrência de uma conversão/degradação/absorção metabólica de componentes carcinogênicos (O'SULLIVAN *et al.*, 1992; KAILASAPATHY & RYBKA, 1997).

A lactose é fermentada a ácido lático pelas bactérias láticas. Bifidobactérias produzem L(+) ácido lático enquanto outras bactérias ácido láticas produzem D(-) ácido lático. Crianças metabolizam totalmente L(+) ácido lático enquanto D(-) ácido lático é apenas parcialmente metabolizado, numa velocidade muita lenta. D(-) ácido lático não metabolizado é excretado pela urina e causa acidose metabólica. Por essa razão as bifidobactérias seriam mais desejáveis na microflora do intestino das crianças (RASIC & KURMANN *apud* LAROIA & MARTIN, 1990).

Apesar de existirem muitas controvérsias a respeito, alguns estudos tem relacionado o abaixamento nos níveis de colesterol sérico com o consumo de leites fermentados. Muitos mecanismos tem sido propostos para explicar esse efeito, entre eles a produção pelas bactérias ácido láticas de hidroximetilglutarato, o qual inibe a hiroximetilglutaril- Coa redutase que está envolvida na síntese do colesterol; a desconjugação de ácidos biliares pelas bactérias probióticas resulta em menor eficiência de absorção do colesterol no intestino; a produção de alguns ácidos orgânicos que atuariam inibindo a síntese do colesterol (LAROIA & MARTIN, 1990; HUGHES & HOOVER, 1991; O'SULLIVAN *et al.*, 1992).

Os principais efeitos benéficos e as aplicações terapêuticas atribuídas as bactérias probióticas são apresentados no Quadro 3.

Quadro 3. Efeitos benéficos e aplicações terapêuticas das bactérias probióticas em humanos.

Efeitos benéficos
Manutenção das microfloras normais do intestino e aparelhos urogenitais
Alívio da intolerância a lactose
Redução dos níveis de colesterol sérico
Atividade anticarcinogênica
Estímulo do sistema imunológico
Melhora do valor nutricional dos alimentos
Aplicações terapêuticas
Prevenir infecções urogenitais
Aliviar constipação
Proteção contra "diarréias de viagem"
Prevenção de diarréias infantis
Redução de diarréias causadas por antibióticos
Prevenção da hipercolesterolemia
Prevenção contra câncer de cólon
Redução dos efeitos da encefalopatia hepática
Auxílio em casos de hino- e hipercorohidria
Prevenção da osteoporose

Fonte: FULLER (1989).

Apesar das inúmeras evidências dos efeitos benéficos das bactérias probióticas (Quadro 3), segundo O'SULLIVAN *et al.* (1992), essas pesquisas precisam de mais suporte científico, uma vez que podem ter ocorrido delineamentos experimentais, análises estatísticas e escolhas das cepas inadequadas, além de falhas no controle de qualidade das culturas e produtos.

Segundo SANDERS (1999), os estudos que documentam os efeitos dos probióticos em humanos ainda são limitados, entretanto resultados de vários sistemas biológicos são intrigantes e o uso comercial de probióticos procede porque essencialmente eles não causam nenhum risco à saúde e muitos

benefícios são incontestáveis, como o controle de diarréias e a melhoria na intolerância à lactose.

5. Condições de sobrevivência

Muitos microrganismos têm sido estudados para serem utilizados como adjuntos, para melhorarem a qualidade nutricional e terapêutica dos alimentos. No entanto, segundo KIM (1988), alguns requerimentos são necessários para se alcançar tal propósito. Assim, esses microrganismos deveriam:

- ser habitantes naturais do trato intestinal, para não provocarem distúrbios no balanço da flora ali presente;
- ser capazes de sobreviver à passagem pelo trato gastrintestinal (suco gástrico e sais biliares);
- colonizar o intestino;
- ser metabolicamente ativos e produzir efeitos benéficos enquanto estão no intestino;
- permanecer viáveis no alimento até o momento do consumo em número de no mínimo 10^6 ufc/ml ou g.

A maioria das bactérias são destruídas quando passam pelo estômago humano em função da presença do suco gástrico que é um dos nossos melhores mecanismos de defesa contra microrganismos patogênicos. A motilidade intestinal e o efeito inibitório dos sais biliares dificultam a colonização do intestino delgado (NAIDU *et al.*, 1999). Muitos estudos investigaram a sobrevivência de *L. acidophilus* e/ou *Bifidobacterium* spp. na presença de ácido e sais biliares (CONWAY *et al.* 1987; BERRADA *et al.* 1991; HOLCOMB *et al.* 1991; CLARK *et al.* 1993; CLARK & MARTIN, 1994; LANKAPUTHRA & SHAH, 1995; MARTEAU *et al.* 1997; CHOU & WEIMER, 1999).

MODLER & VILLA-GARCIA (1993) reportaram que algumas espécies de bifidobactérias demostraram ácido-tolerância em pH 4,0. CLARK *et al.* (1993) também reportaram a sobrevivência de algumas espécies de *B. infantis, B.*

adolescentis e *B. longum* nos pHs 2 e 3, apesar do número de células viáveis ter diminuído rapidamente em pH 1.

L. acidophilus mostraram-se mais resistentes às condições adversas do que *B. bifidum*. Sobreviveram em grande número no pH 3,9 - 4,6 (LAROIA & MARTIN, 1991).

LANKAPUTHRA & SHAH (1995) testaram a sensibilidade de algumas cepas de *L. acidophilus* e *Bifidobacterium* spp. a diferentes pHs (1,5; 2,0; 2,5 e 3) e concentrações de bile (0, 1 e 1,5%) durante períodos de incubação de 0, 1, 2 e 3 horas. As cepas 2401, 2409 e 2415 de *L. acidophilus* sobreviveram melhor (10^8 UFC/g) sob condições ácidas, enquanto as cepas 2404 e 2415 apresentaram melhor tolerância à bile (10^7 a 10^8 UFC/g) após 3 horas de exposição. Entre as linhagens de *Bifidobacterium* spp., *B. longum* 1941 e *B. pseudolongum* 20099 sobreviveram melhor (10^8 UFC/g) sob condições de baixa acidez e *B. longum* 1941, *B. pseudolongum* 20099 e *B. infantis* 1912 mostraram melhor tolerância à bile (10^8 a 10^9 UFC/g).

LANKAPUTHRA *et al.* (1996) testaram a viabilidade de 9 espécies de bifidobacteria em leite acidificado nos pHs 4.3, 4.1, 3.9 e 3,7, armazenado sobre refrigeração durante 42 dias e verificaram que apenas três espécies *B. infantis* 1912, *B. longum* 1941 e *B. pseudolongum* 20099 foram capazes de sobreviver em bom número, as demais (*B. bifidum* 1900 e 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 e *B. thermophilum* 20210) foram exterminadas em razão do pH baixo ou do pH baixo e H_2O_2.

CLARK & MARTIN (1994) também avaliaram a tolerância *de B. bifidum, B. infantis, B. adolescentis* e *B. longum* à bile (0, 2 e 4%) por um período de 12 horas. Decorrido o período de incubação, *B. longum* mostrou-se o mais tolerante, com taxa de sobrevivência de cerca de 10^7 UFC/ml, em 2% de bile. As demais espécies não sobreviveram após 12 horas de exposição, nas concentrações de 2 e 4%. Entretanto, é preciso considerar que o tempo e concentração de bile foram drásticos, uma vez que a concentração máxima de bile no intestino delgado humano é de 2% e que tem sido demonstrada uma redução nessa concentração para cerca de 0,5% após a segunda hora de ingestão do alimento.

6. Incorporação em iogurte

A viabilidade da incorporação de bactérias probióticas em iogurte depende de uma série de fatores intrínsecos e extrínsecos entre eles as cepas usadas, interações entre as espécies presentes, condições da cultura, composição química do meio de fermentação (por exemplo, fonte de carboidrato), acidez final do produto, teor de sólidos no leite, disponibilidade dos nutrientes, presença de promotores e inibidores de crescimento, concentração de açúcar (pressão osmótica), oxigênio dissolvido (especialmente para bifidobactérias), nível e temperatura de inoculação, tempo de fermentação e temperatura de estocagem (KAILASAPATHY & RYBKA, 1997; SHAH, 2000).

Segundo TAMINE & ROBINSON (1985), a incorporação de *L. acidophilus* e *B. bifidum* em iogurte resulta em um produto de excelente valor terapêutico. Quanto à qualidade sensorial, o produto normalmente apresenta aroma e sabor suaves e viscosidade e consistência inferiores aos produtos tradicionais (LAROIA & MARTIN, 1990).

L. acidophilus e *Bifidobacterium* spp. isolados crescem muito pouco no leite não enriquecido durante a manufatura de produtos fermentados, entretanto, a prática usual é utilizar esses microrganismos em associação com outros microrganismos como *L. delbrueckii* spp. *bulgaricus* e *S. salivarus ssp. thermophilus* ou já partir de concentrações maiores de inóculo (LAROIA & MARTIN, 1990; SHAH, 2000). Segundo BADRAN & REICHART (1995), a utilização de *B. bifidum*, *L. acidophilus* e *L. bulgaricus* em associação levou a um maior crescimento do que quando essas culturas foram utilizadas isoladamente. Há um efeito sinergístico de crescimento entre *B. bifidum* e *L. acidophilus* (KNEIFEL *et al.* 1993) além do mais, a combinação dessas culturas é bastante interessante uma vez que o primeiro coloniza e portanto atua efetivamente no intestino grosso e o segundo no fino. (LAROIA & MARTIN, 1991). Entretanto, *L. delbrueckii* spp. *bulgaricus* produz ácido lático durante a fermentação e na estocagem refrigerada. A produção de ácido durante a estocagem é um processo

conhecido como pós-acidificação e foi responsabilizado pela redução na viabilidade das culturas probióticas incorporadas aos iogurtes comerciais (SHAH *et al.* 1995).

Dois estudos mostraram que a utilização de *B. bifidum* com a cultura de iogurte (proporção 2:1) resultou em uma grande mudança nos componentes nitrogenados, com melhora na digestibilidade das proteínas, em relação ao produto onde foi utilizada a cultura de iogurte sozinha. Esse resultado foi atribuído à atividade proteolítica de *B. bifidum* no leite (GOODENOUGH & KLEYN, 1976; RASIC & KURMANN *apud* LAROIA & MARTIN 1990).

A estocagem refrigerada afetou a sobrevivência dos microrganismos presentes em um produto comercial a base de leite fermentado. Após 24 dias de estocagem as populações de *L. delbrueckii* spp. *bulgaricus, S. salivarus ssp. thermophilus* e *Bifidobacterium* ssp. diminuíram 10,7, 85,4 e 92,6%, respectivamente (MEDINA & JORDANO, 1994). Em contraste, quando *B. longum*, *B. breve* e *B. infantis* foram utilizados como adjuntos em sorvete, após 70 dias de estocagem a -17°C, 90% dos microrganismos sobreviveram, sendo que a perda (de 10%) ocorreu em virtude da incorporação de ar (MODLER *et al.*, 1990a).

O pH baixo dos produtos fermentados é um fator extremamente limitante para o desenvolvimento das bifidobactérias. *B. bifidum* não sobreviveu em um produto fermentado onde o pH variava de 3,9 a 4,6 (LAROIA & MARTIN, 1991). A capacidade de sobreviver em iogurte de baixa ou alta acidez sob estocagem refrigerada varia entre as espécies/linhagens de bifidobactéria. Algumas linhagens foram capazes de sobreviver em iogurte de alta acidez por meses, enquanto outras não sobreviveram nem um mês, mesmo em iogurte de baixa acidez (MARTIN & CHOU, 1992).

A acidificação que ocorre durante a estocagem, devido ao crescimento incontrolado de *L. delbrueckii* subsp. *bulgaricus,* à temperatura de refrigeração, reduz rapidamente a contagem das células viáveis, inclusive de *B. bifidum*, em iogurte (MARSHALL, 1991; RYBKA *apud* KAILASAPATHY & RYBKA, 1997), por outro lado, foi detectado um efeito estimulatório de crescimento, na associação dessas culturas (*L. delbrueckii* ssp. *bulgaricus* e *B. bifidum)* provavelmente devido

à atividade proteolítica, produção de valina, glicina e histidina (MISRA & KUILA, 1994).

7. Meios para contagem

Monitorar o número de células viáveis de microrganismos probióticos nos alimentos em que os mesmos são incorporados é um parâmetro fundamental para assegurar a qualidade do produto que é comercializado com apelo terapêutico, entretanto, muitas vezes essa necessidade tem sido negligenciada em função da falta de padronização de métodos.

Os meios para enumeração específica de bifidobactérias em geral são caracterizados pela presença de substâncias capazes de reduzir o Potencial de oxi-redução (por exemplo, cistina, cisteína, ácido ascórbico e sulfito de sódio), antibióticos, uma fonte simples de carbono e agentes seletivos para inibir o crescimento de outras bactérias ácido láticas, e são freqüentemente fortificados com sangue de cavalo ou carneiro (RASIC, 1990). As condições de incubação são: anaerobiose, temperatura entre 37 e 41°C e por tempo $\geq$ 48 horas (CHARTERIS *et al.*, 1997).

O Ágar NPNL é um meio seletivo para espécies de bifidobactérias, desenvolvido por TERAGUCHI et al. (1978), que apresenta em sua composição os compostos inibidores: sulfato de neomicina, paramomicina, ácido nalidíxico e cloreto de lítio. Este meio foi utilizado em inúmeras pesquisas (LAROIA & MARTIN, 1991; MARTIN & CHOU, 1992; CLARK et al., 1993; CLARK & MARTIN, 1994).

SAMONA & ROBINSON (1991) avaliaram quatro meios seletivos e seis não seletivos para contagem de bifidobactérias. Entre os meios examinados destacou-se o meio ágar Rogosa modificado, que possibilitou o melhor crescimento das bifidobactérias. Porém, os resultados não foram efetivamente conclusivos.

Os meios ágar NPNL, ágar bile, ágar MRS-galactose, ágar MRS-galactose, ágar MRS-maltose, ágar MRS-dextrose, ágar MRS-L-arabinose e RCA foram avaliados com o intuito de determinar a capacidade seletiva para 6 cepas de *L.*

acidophilus, 9 de *Bifidobacterium*, 5 de *L. delbrueckii* spp. *bulgaricus* e 6 de *S. salivarius* spp. *thermophilus*. O Ágar NPNL permitiu o crescimento de 6 espécies de bifidobactérias e inibiu o crescimento das demais culturas. Entretanto, este meio não foi efetivo para contagem das espécies *B. infantis* 1912, *B. adolescentis* 1920 e *B. thermophilus* 20210 haja visto que não permitiu o crescimento das mesmas. O ágar bile foi considerado eficiente para contagem seletiva das espécies de *L. acidophilus* utilizadas na presença de *B. bifidum*, *B. adolescentis*, *B. breve* e *B. longum* 20097, ou seja, ele inibiu o crescimento de algumas linhagens de *Bifidobacterium,* mas permitiu o crescimento de outras (LANKAPUTHRA *et al.,* 1996).

ONGOO & FLEET (1993) avaliaram dois meios de diferenciação: Ágar Triptose Proteose Peptona Extrato de levedura (TPPY) e ágar Reinforced Clostridial acrescido de Prussian blue (RCPB). As espécies de *L. delbrueckii* spp. *bulgaricus, S. salivarius* spp. *thermophilus* e *B. bifidum* foram facilmente diferenciadas em ágar RCPB devido às características distintas das suas colônias. As espécies de *L. delbrueckii* spp. *bulgaricus, S. salivarius* spp. *thermophilus* e *L. acidophilus* também puderam ser diferenciadas em ágar TPPY em função das características distintas das suas colônias.

DAVE & SHAH (1996) testaram 15 meios para avaliar a eficiência destes para enumeração seletiva da cultura de iogurte, *L. acidophilus e* bifidobactéria, utilizando 5 a 6 linhagens de cada grupo de microrganismo. Ágar *Streptococcus thermophilus* (ST) possibilitou a enumeração seletiva de *S. thermophilus* sob condições anaeróbicas, a 37^{0}C, por 24 horas. Ágar MRS com pH ajustado para 5.2 ou RCA pH 5.3 podem ser utilizados para contagem de *L. delbrueckii* spp. *bulgaricus* quando a incubação é feita a 45°C, por 72 horas. Ágar MRS-maltose pode ser utilizado para a enumeração em conjunto de *L. acidophilus* e bifidobactérias. Para a enumeração seletiva de *L. acidophilus* ágar MRS- salicina ou ágar MRS-sorbitol poderiam ser usados. Para enumeração seletiva de bifidobactérias ágar MRS-NPNL foi o mais indicado.

ZACCONI *et al.* (1990) reportaram a eficiência do meio ágar MRS acrescido de dicloxacilina e cloridato de cisteína para contagem seletiva de bifidobactéria em

produtos lácteos fermentados. GRENOV citado em SHAH (1997) e DE STEFANO *et al.* (2000), utilizaram com sucesso o meio seletivo: MRS acrescido de dicloxacilina, cloridato de cisteína e cloreto de lítio para contagem de bifidobactéria na presença das culturas do iogurte.

É crescente o consenso de que alguns meios que contêm bile e antibióticos poderiam também restringir o crescimento de *L. acidophilus* e bifidobactérias e que a contagem total obtida nestas condições, nem sempre é representativa do verdadeiro número de células viáveis presentes no produto (DAVE & SHAH, 1996).

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THE EFFECT OF THE IMMOBILIZATION OF *L. acidophilus & B. lactis* IN ALGINATE ON THEIR TOLERANCE TO GASTROINTESTINAL SECRETIONS

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Summary

This research aimed at evaluating the "in vitro" tolerance of *Bifidobacterium lactis* and *Lactobacillus acidophilus*, both free and immobilized in calcium alginate, to pH and bile levels similar to those encountered in the human stomach and intestine, respectively. Free and immobilized cultures were inoculated, at the level of 10%, into HCl solutions with pH values of 1 and 2, and then incubated anaerobically at 37°C, being subsequently plated out at intervals of 0, 1 and 2 hours after incubation. The bile concentrations tested were 0, 2 and 4%. Counts were made by the pour plate method using *MRS broth* after 0 and 12 hours of anaerobic incubation. Morphological observations of the cells immobilized in alginate gel were made using a scanning electron microscope. The cell counts of samples containing alginate drops were effected after solubilization in 2% sodium citrate using a *Stomacher* homogenizer. A pH value of 2 promoted a slight decline in the number of viable microorganisms and pH 1 was extremely deleterious for both *B. lactis* and *L. acidophilus*, the same effect being shown for the cells immobilized in alginate. Innoculation into bile did not affect the stability of *B. lactis* and *L. acidophilus.* Thus *B. lactis* and *L. acidophilus* showed intolerance to pH 1 and immobilization in alginate was not effective in protecting their cells.

1. Introduction

FULLER (6) defined probiotics as "a food supplement of live microrganisms, which benefits the host improving the intestinal microbial flora". Many papers have recounted the use of probiotic microrganisms as dietary aids in foods (15, 16, 19). However, according to KIM *et al.* (12), certain requirements are necessary to attain this objective: the microrganism used should be a natural inhabitant of the intestine; should be capable of colonizing the intestine; should be metabolically active to produce the beneficial effect; must remain viable in the food up to the moment of consumption and be capable of surviving its passage through the

gastrointestinal tract. The bacteria *Lactobacillus acidophilus, L. casei, Bifidobacterium lactis* (previously nominated *B. bifidum*), *B. longum* and the yeast *Saccharomyces boulardii* are the organisms most widely used as probiotics in humans (17).

Some papers mention the intolerance of certain strains of *B. lactis* and *L. acidophilus* to stomach acidity and/or to bile (1, 2, 3, 4, 13 and 14). Immobilization in alginate was used with the objective of protecting the cells of *B. lactis, B. infantis* and *B. longum* which were added to "crescenza" cheese (7), of *B. lactis* and *B. infantis* added to maionnaise and of *Lactobacillus bulgaricus* incorporated into ice-cream (20). *Bifidobacterium pseudolongum*, microencapsulated in cellulose acetate phthalate was more resistant than the free cells when innoculated into a pH similar to that of the stomach (18).

This research evaluated the tolerance of *Bifidobacterium lactis* and *Lactobacillus acidophilus,* both free and encapsulated in calcium alginate, to pH and bile levels similar to those encountered in the human stomach and intestine, respectively.

2. Materials and Methods

2.1 Culture Maintenance

Pure freeze dried cultures of *Lactobacillus acidophilus* (La-05) and *Bifidobacterium lactis* (Bb-12) kindly provided by Cr. Hansen of Brazil, were maintained in the proportion of 1g per 100 mL of 12% reconstituted non fat dryer milk (NFDM) at a temperature of -18°C.

2.2 Immobilization

The immobilization of *Lactobacillus acidophilus* and *Bifidobacterium lactis* in calcium alginate was carried out by coextrusion following the methodology summarized in Fig. 1. One per cent sodium alginate (Manugel - DMB-Nutrasweet/Kelco) was used, previously sterilized at 121°C for 15 minutes. The mixture (alginate-milk) was added drop by drop to a sterile 2% solution of $CaCl_2$

using a peristaltic pump (Cole Parmer Instrument Co. - model # 7553-20). The drops remained in the $CaCl_2$ overnight to harden. The whole procedure was carried out under sterile conditions in a laminar flow chamber.

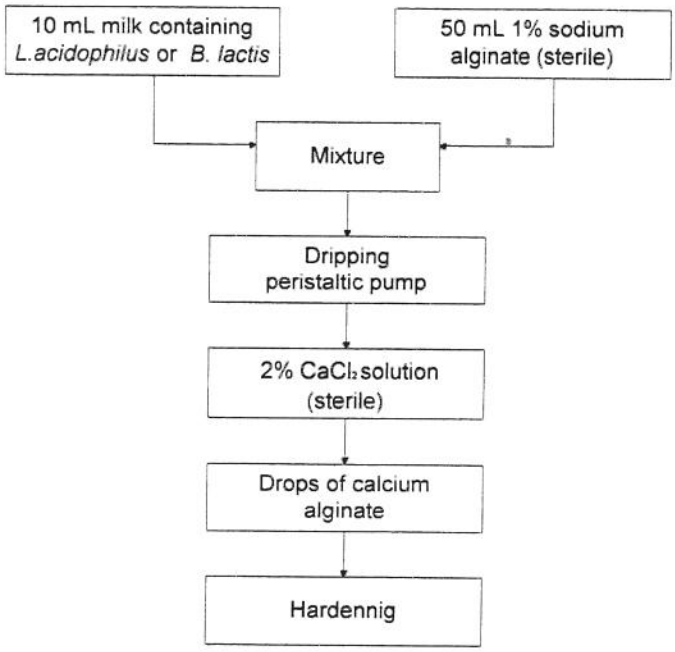

Fig. 1: Flowsheet of the immobilization of *L. acidophilus* and *B. lactis* in calcium alginate.

2.3 Survival of the bacteria at pH levels similar to those of the human stomach

The determination of the effect of pH values similar to those of the stomach on free and immobilized cells of *L. acidophilus* and *B. lactis* was carried out according to CLARK *et al.* (2), using distilled water at pH 7.2 as the control.

2.4 Survival of the bacteria at bile levels similar to those of the human intestine

This test was carried out according to the methodology described by CLARK & MARTIN (3), using Oxgall (Difco) for the bile solutions and peptone water as the control.

2.5 Colony counts

DeMan Rogosa and Sharp (MRS) broth (Oxoid) was used to enumerate both cultures by the pour plate technique. A 2% solution of sodium citrate was used to make serial dilutions from 10^1 to 10^8. One mL of each dilution was transferred to a petri plate and melted MRS at 49°C poured on and mixed by swirling. After solidification, the plates were inverted and incubated anaerobically at 37°C for 72 h in anaerobic jars with the Anaerogen (Oxoid) system for generating anaerobic conditions. Plating was carried out in duplicate.

2.6 Solubilization of alginate drops

In order to carry out a quantitative analysis of the viable cells by the pour plate method, it was necessary to solubilize the alginate drops in order to liberate the immobilized cells. This was done using sterile 2% sodium citrate in a Stomacher homogenizer for two minutes at normal velocity and environmental temperature.

2.7 Size and morphology of the alginate drops

The diameter of the alginate drops was measuresd with the aid of a Mitutoyo digital micrometer (Model SR44). One hundred alginate drops were measured.

Scanning electron microscopy was used to examine the arrangement of the cells within the alginate drops and the appearance of the gel. The drops were sliced using a blade and then dried in a Balzers Critical Point Dryer equipment (Model CPD 030). The dried specimens were mounted on an aluminium stub and coated with a fine layer of gold in a Balzers evaporator (Model SCD 050) for 120 s using a current of 40 mA. Microscopy was performed in a JEOL Scanning Electron Microscope (Model JSM-T300) at an accelerating voltage of 20 KV.

2.8 Statistical analyses

The ANOVA analysis of variance and Tukey's test were carried out to determine if significant reductions in the number of viable cells of *B. lactis* and *L.*

acidophilus occured during the period of incubation in bile. The STATISTICA 6.0 software (Microsoft®) was used and the experiments were carried out in triplicate.

3. Results

3.1 Size and morphology of the alginate drops

The alginate drops presented an average diameter of 2.37 mm with a standard deviation of ±0.20, in agreement with the results of DALILI & SHAU (5), who obtained alginate drops by coextrusion with diameters varying between 2 and 4 mm. Figure 2 shows *B. lactis* cells immobilised in calcium alginate gel, the figure showing a uniform distribution of the cells in the gel. The morphology of the gel is in accordance with that observed by SHEU *et al.* (20).

3.2 Effect of pH values of 1 and 2 on the survival of B. lactis and L. acidophilus.

Both the free and immobilized cells of *B. lactis* were shown to be relatively resistant to pH 2. Both showed a reduction of one logarithmic cycle in the population count after one hour of incubation (Fig. 3 and 4).

Free *B. lactis* was shown to be intolerant of a pH value of 1 right from the moment of exposure, there being an immediate reduction of two logarithmic cycle in the population at zero time when compared to the control. After one hour at this pH, the entire population was eliminated, the same occurring with the immobilised cells, except that at zero time the population was not affected as compared to the control (Figs. 3 and 4). This result agrees with that of CLARK *et al.* (2).

Practically no alteration in the population of *L. acidophilus* was shown during the whole incubation period at pH 2.0 (Fig. 5), in contrast to the result obtained by HOOD & ZOTTOLA (10) who observed a considerable decline in the count of *L. acidophilus* 45 minutes after inoculation at pH 2.0.

L.acidophilus also presented intolerance to pH 1.0. It can be seen in Fig. 5 that after 1 hour of incubation, the population reduction was to the order of 6 cycles, that is, the whole population had been decimated. The immobilized cells of

L. acidophilus were also totally eliminated after 1 hour of incubation at pH 1 (Fig. 6).

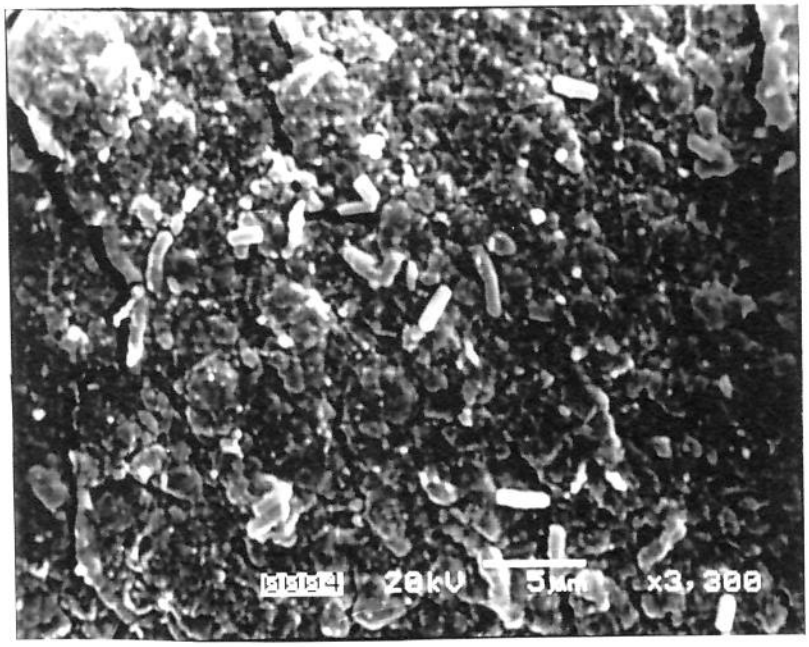

Fig. 2: *B. lactis* immobilized in calcium alginate gel.

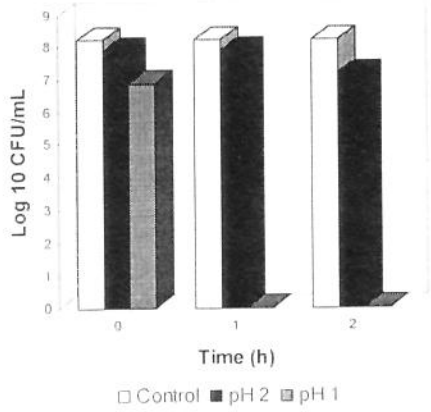

Fig. 3: Effect of pH on the survival of free *B. lactis*.

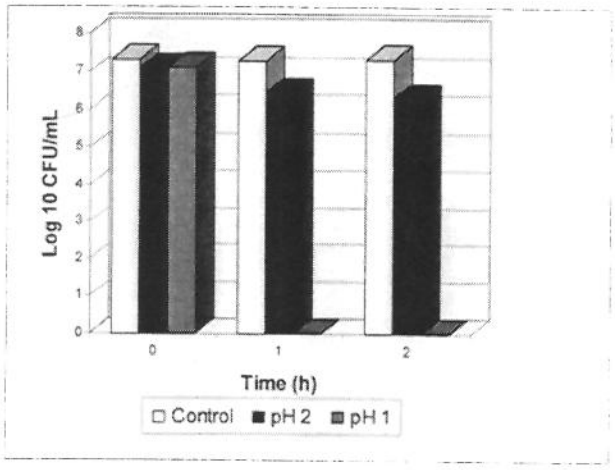

Fig. 4: Effect of pH on the survival of immobilized *B. lactis.*

Immobilization was shown to be effective for both *B. lactis* and *L. acidophilus* only at zero time at pH 1, under which conditions the populations were practically unaltered (Figures 4 and 6), whereas under the same conditions the free cells were affected (Figures 3 and 5). The lack of efficiency of immobilization during a longer period was probably due to the porosity of the polymannuronic alginate gel (11), allowing for the diffusion of H^+ ions into the interior of the alginate drop and thus affecting the cells.

The results obtained in this experiment do not differ from those obtained by CONWAY *et al.* (4), in which the populations of two strains of *L. acidophilus* were reduced by 6 logarithmic cycles after 1 hour of incubation in phosphate buffer at pH 1, and were also affected, although less intensely, by pH values of 3 and 5. On the other hand, MARTEAU *et al.* (14) reported the survival of more than 40% of the populations of *L. acidophilus* and *B. bifidum* (*B. lactis*) in a system similar to that of the gastric compartment, after 120 minutes of incubation. Contradictory results can be explained, since according to LANKAPUTHRA & SHAH (13), for both *L. acidophilus* and *Bifidobacterium* spp., the resistance to acid pH values and to the presence of bile can greatly vary between different strains of the same species. This confirms the need to identify those strains of probiotic microrganisms

tolerant to gastrointestinal secretions, which could therefore be used as dietary aids.

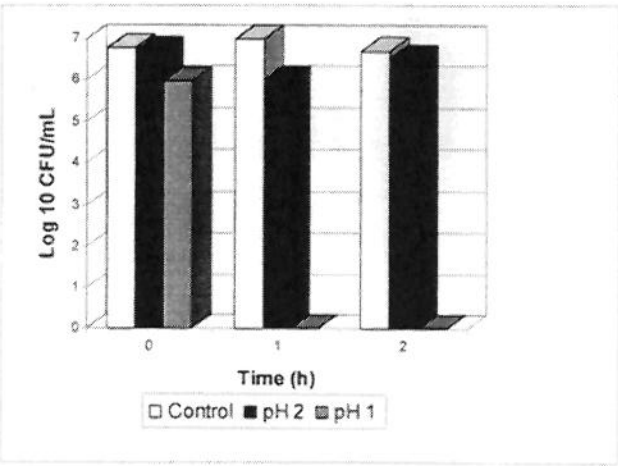

Fig. 5: Effect of pH on the survival of free *L. acidophilus*.

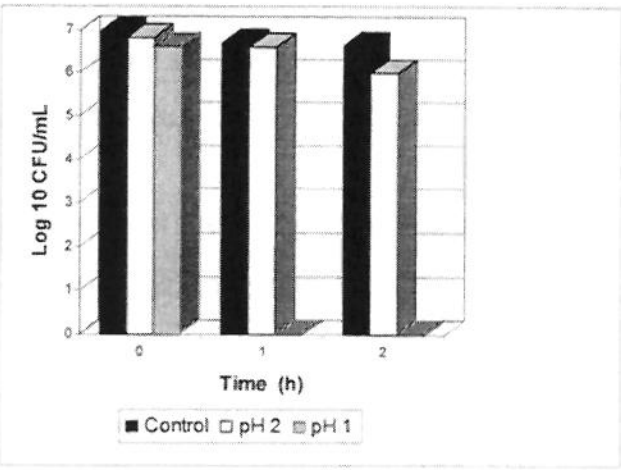

Fig. 6: Effect of pH on the survival of immobilized *L. acidophilus*.

3.3 Effect of the inoculation of bile on the survival of B. lactis and L. acidophilus

The survival of *B. lactis* and *L. acidophilus* was evaluated in the presence of 2 and 4% bile during 12 hours of incubation, according to the results presented in Tables 1 and 2.

It can be seen in Table 1 that there was no significant ($p<0.05$) reduction in the populations of either free or immobilized *B. lactis* as compared to the control, at either of the bile concentrations tested, even after 12 hours of incubation, showing that *B. lactis* is extremely resistant to the presence of bile, independant of being free or immobilized. This result is in accordance with that of HOLCOMB *et al.*(9) who, despite testing lower concentrations of bile, observed that these did not affect the numbers of *B. bifidum* (*B. lactis*). On the other hand, our results differ from those of CLARK & MARTIN (3) in which the population of *B. bifidum (B. lactis)* was completely decimated after 12 hours of incubation in 2% bile or in more concentrated solutions.

Table 1: Survival of free and immobilized *B. lactis* during 12 hours after inoculation into bile.

	Bile concentration (%)		
Condition/Time (h)	0 (control)	2	4
Free / 0	6.0×10^{7Aa}	6.5×10^{7Aa}	9.2×10^{7Aa}
Free / 12	2.0×10^{7Aa}	6.9×10^{7Aa}	9.0×10^{7Aa}
Immobilized / 0	1.4×10^{7Aa}	2.1×10^{7Aa}	2.1×10^{7Aa}
Immobilized / 12	1.7×10^{7Aa}	2.0×10^{7Aa}	2.1×10^{7Aa}

[A] Means with the same letter in the same line are not significantly different ($P<0.05$).
[a] Means with the same letter in the same column are not significantly different ($P<0.05$).

Inoculation into bile also had no effect on the population of *L. acidophilus*, as can be seen in Table 2, there being no significant reduction in the cell count as compared to the control in either of the conditions tested.

B. lactis and *L. acidophilus* thus demonstrated their potential for survival and growth in the intestine, due to their bile tolerance, this being a requirement for their use as probiotics.

Table 2: Survival of free and immobilized *L. acidophilus* during 12 hours after inoculation into bile.

	Bile concentration (%)		
Condition/Time (h)	0 (control)	2	4
Free / 0	9.1×10^{6Aa}	8.3×10^{6Aa}	5.7×10^{6Aa}
Free / 12	9.2×10^{6Aa}	2.5×10^{6Aa}	6.3×10^{6Aa}
Immobilized / 0	8.6×10^{6Aa}	4.3×10^{6Aa}	3.6×10^{6Aa}
Immobilized / 12	8.1×10^{6Aa}	5.1×10^{6A}	3.3×10^{6Aa}

[A] Means with the same letter in the same line are not significantly different ($P<0.05$).
[a] Means with the same letter in the same column are not significantly different ($P<0.05$).

4. Conclusions

Lactobacillus acidophilus (La-05) and *Bifidobacterium lactis* (Bb-12) showed good potential for consumption as dietary aids due to their bile tolerance and relative resistance to pH 2, although the therapeutic benefits would be limited if the stomach pH of the consumer were close to 1. Thus the choice of strains resistant to both acid and bile, or of means to protect them from the effect of these secretions, is of fundamental importance in the elaboration of food to be consumed for therapeutic purposes.

Acknowledgements

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THE STABILITY OF *Lactobacillus acidophilus* & *Bifidobacterium lactis* IN THEIR FREE AND IMMOBILISED FORMS, IN ACIDIFIED MILK AND YOGHURT DURING REFRIGERATED STORAGE

Summary

This study evaluated the stability of *B. lactis* and of *L. acidophilus* in both the free form and immobilised in calcium alginate, in acidified milk, and the stability of immobilised *B. lactis* in yoghurt. It also determined the efficiency of two culture mediums in the counting of *B. lactis* in yoghurt, one being selective and the other differential. The stability of both free and immobilised *L. acidophilus* and *B. lactis* incorporated in milk and acidified milk, was determined at pH values of 5.0, 4.4 and 3.8 after 0, 7, 14, 21 and 28 days of refrigerated storage. The viability of immobilised *B. lactis* in yoghurt, previously fermented to pH 4.2, was determined during 28 days of refrigerated storage. Morphological observations of the cells were made using an optical microscope. Counts of *B. lactis* in yoghurt were made using the following media: MRS agar supplemented with dicloxacillin, lithium chloride, cysteine and aniline blue (selective) and the differential agar RCA plus added Prussian Blue (RCPB). Lee's agar was used to count *Streptococcus salivarus* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* when *B. lactis* was counted in the selective medium. Both microorganisms in both the free and immobilised forms, presented good rates of survival in acidified milk. Immobilised *B. lactis* showed poor viability in yoghurt and the selective medium was more efficient for the counting of this organism in yoghurt, when the cultures were not in equilibrium.

Introduction

FULLER (1989) defined probiotic as a "food supplement composed of live microbial cells, which show beneficial effects for the host by improving the microbial equilibrium in the intestine". *Lactobacillus acidophilus, L. casei, Bifidobacterium bifidum, B. longum* and *Saccharomyces boulardii* are frequently used as probiotics in products for humans (PLAYNE, 1994), although other species are also recognised as probiotics.

Foods containing these microorganisms are being sold in many countries, although their survival in foods is doubtful, since some of the strains are extremely

sensitive to a series of factors. Also, methods for counting these organisms have not yet been well established, which is an essential requirement to determine their survival in commercial products (KAILASAPATHY & RYBKA, 1997).

The survival of *L. acidophilus* and *Bifidobacterium* spp. in yoghurts has been shown to be a problem, due to their intolerance of acid conditions and the presence of other cultures, such as *L. delbrueckii* ssp. *bulgaricus* (LAROIA & MARTIN, 1991; MODLER & VILLAGARCIA, 1993). However, microencapsulation techniques could provide protection to acid sensitive bifidobacterias and thus increase their survival rate during the shelf life of the yoghurt and during their passage through the gastrointestinal tract (RAO *et al.*, 1989; MODLER *et al.* , 1990).

The immobilisation of *Lactobacillus bulgaricus* in calcium alginate offered good protection to the organisms during frozen storage and in ice cream (SHEU & MARSHALL, 1993). Bifidobacterias immobilised in alginate were more resistant to acid pH values in mayonnaise than the free cells (KHALIL & MANSOUR, 1998).

The objectives of this study were: to evaluate the stability of *B. lactis* and *L. acidophilus,* both free and immobilised in calcium alginate, in acidified milk, determine the stability of *B. lactis* in yoghurt, and to verify the efficiency of two culture media, one selective and the other differential, in counting *B. lactis* in yoghurt.

Material & Methods

Cultures

Lactobacillus acidophilus (La-05), *Bifidobacterium lactis* (Bb-12) and a mixed culture of *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Chr. Hansen, Valinhos, Br.), in the DVS (direct vase set) form, pure and freeze dried, were maintained in the proportion of 1g per 150 ml in a sterile solution of 12% reconstituted skimmed milk, at a temperature of -18°C. The mixed culture was replicated twice before use, using an initial inoculum of 2% in 12% sterile skimmed milk, at a temperature of 45°C for 3 hours.

Streptococcus salivarius ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, isolated, from Wisby labor., were used in the evaluation of the culture media. For this, they were replicated in sterile 12% skimmed milk, at a temperature of 45°C for 3 hours.

Culture counts

In the absence of other cultures, *L. acidophilus* and *B. lactis* were counted in DeMan Rogosa and Sharp (MRS) broth (Oxoid) by the pour plate technique. A 2% solution of sodium citrate was used to make serial dilutions from 10^1 to 10^8. One mL of each dilution was transferred to a petri plate and melted MRS at 49°C poured on and mixed by swirling. After solidification, the plates were inverted and incubated anaerobically at 37°C for 72 h in anaerobic jars with the Anaerogen (Oxoid) system for generating anaerobic. Plating was carried out in duplicate.

For quantitative measurements of the number of viable cells, it was necessary to solubilize the alginate drops to liberate the microorganisms. This was effected in 2% sterile sodium citrate solution, using the Stomacher 400 (Seward, London, UK), at medium velocity and room temperature, for 2 minutes.

Evaluation of the culture media for counting *B. lactis* in yoghurt

Two media were evaluated for the counting of *B. lactis* in the presence of yoghurt cultures, one differential and the other selective.

Selective: MRS agar – deMan, Rogosa and Sharpe Broth (Oxoid, Hampshire, UK) supplemented with: 0.5% of a 10% solution of L-cysteine hydrochloride (Synth, São Paulo, Brazil), 0.5% of a dicloxacillin solution (10mg/100ml water) (Sigma, Louis, USA) and 1% of a 10% solution of lithium chloride (Vetec, São Paulo, Br) (Grenov *apud* Shah, 1997; De Stefano, 2000), modified by addition of 0.01% aniline blue (Nuclear, São Paulo, Brazil). For this the spread plate technique was used, incubating in jars containing the Anaerobac (Probac, São Paulo, Br) anaerobiosis generating system, at 37°C, for 72 h.

Lee's agar (LEE *et al.*, 1974) was formulated and used to count *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp.

bulgaricus, when *B. lactis* was counted in the selective medium. In this case the spread plate technique was used, incubating in jars containing the Microaerobac (Probac, São Paulo, Br) microaerophyllic generating system, at 37°C, for 48 hours.

Differential: RCPB composed of RCA – Reinforced Clostridial Agar (Oxoid, Hampshire, UK) with added 0.03% Prussian Blue (Aldrich, USA) (ONGGO & FLEET, 1993). The conditions for plating and incubation were similar to those used with the MRS medium.

Immobilisation process

The immobilisation of *L. acidophilus* and *B. lactis* was carried out according to FÁVARO-TRINDADE & GROSSO (2000).

Optical microscopy

The optical microscope model Jenaval (Zeiss) was used, with an immersion objective of ×100, optovar with magnification of 1.25, chamber factor of ×3.2 and 35 mm films.

Evaluation of the stability of immobilised *L. acidophilus* and *B. lactis* in acidified milk

Model systems were elaborated to study the viability of incorporating immobilised *L. acidophilus* and *B. lactis* into acid foods. For this, milk (pH 6.4) was used as the standard and other samples of milk were acidified by the addition of 4N lactic acid to pH values of 5.0, 4.4 and 3.8. All the milk samples used were standardised at 15% solids and sterilised at 121°C for 10 minutes. *L. acidophilus* and *B. lactis*, both free and immobilised in calcium alginate, were added to these milks at a rate of 5%, and stored for 28 days in a BOD incubator (model TE 390, Tecnal, Piracicaba, Brazil) at a temperature of 7°C. Counts were made after 0, 7, 14, 21 and 28 days. This study was carried out in triplicate.

Evaluation of the stability of free and immobilised *B. lactis* in yoghurt

Yoghurt was prepared from whole milk powder, reconstituted at 15% solids, and sterilised at 121°C for 10 minutes. Fermentation was carried out with a 2% inoculum (mixed culture of *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus)* at a temperature of 45°C until a pH of 4.2 was reached. The yoghurt was blended for 5 minutes. The blended product was transferred to plastic cups and immobilised *B. lactis* added at a rate of 2.5% with respect to the volume of yoghurt. The contents were gently mixed with a spatula and the cups capped and stored in a BOD incubator (model TE 390, Tecnal, Piracicaba, Brazil) at a temperature of 7°C for 28 days. With the objective of determining the stability of the immobilised *B. lactis* in the yoghurt, counts were made of both this organism and of *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* after 0, 7, 14, 21 and 28 days of storage. This experiment was carried out in duplicate.

Statistical analysis

To determine the existence of significant differences between the values obtained, a means difference analysis was used according to the Tukey Test, with the aid of the STATISTICA 6.0 software (Microsoft®).

Results

Microscopy

L. acidophilus appeared in the form of short chains of rods (Figure 1). *B. bifidum* appeared as small rods of varying size and showing pleomorphism, that is, variable forms, including the Y shape which can be observed in Figure 2.

Evaluation of culture media for the counting of *B. lactis* in yoghurt

S. salivarius ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and *B. lactis* were easily differentiated in RCPB by the distinct morphologies of their colonies. *L. delbrueckii* ssp. *bulgaricus* grew forming colonies with diameters of from 2 to 3mm, each with a small white clearly defined centre surrounded by a relatively large blue halo; *S. salivarius* ssp. *thermophilus* grew forming colonies

with white centres (less clearly defined than those of *L. delbrueckii* ssp. *bulgaricus*) and a blue halo with a diameter of about 1 mm; *B. lactis* formed very small cylindrical white colonies (approximately 0.5 mm in diameter), as can be seen in Figure 3. This result was very similar to that reported by ONGOO & FLEET (1993), with the difference that these authors obtained larger colonies for *S. salivarius* ssp. *thermophilus* than for *L. delbrueckii* ssp. *bulgaricus.* This difference could have been due to the use of strains from different origins.

It can be seen from Table 6 that, in the yoghurt analyses, when the number of viable cells of *B. lactis* decreased (after 14 days of storage), the count of this organism in RCPB became impossible since the colonies of *S. salivarius* ssp. *thermophilus and L. delbrueckii* ssp. *bulgaricus*.dominated the whole plate.

When the supplemented MRS medium was used for the isolated plating of *S. salivarius* ssp. *thermophilus, L. delbrueckii* ssp. *bulgaricus* and *B. lactis,* previously replicated in milk until coagulation occurred, at dilutions from 10^{-1} to10^{-8}, it was shown that *B. lactis* grew in adequate numbers, forming small brilliant blue colonies with diameters of approx. 0.5 mm, the areas immediately round the colonies being more blue than the rest of the medium (Figure 4), whilst *L. delbrueckii* ssp. *bulgaricus* failed to grow in any of the dilutions plated and *S. salivarius* ssp. *thermophilus* showed limited growth in numbers much lower than expected for the 10^{-1} to 10^{-3} dilutions, forming small light blue colonies with diameters of approx. 0.2 mm, much lighter in colour than *B. lactis,* and with no surrounding blue area, as observed for *B. lactis.*

In the plates containing mixtures of the three colonies in the yoghurt analyses, the supplemented MRS was efficient as a selective medium for the counting of *B. lactis* in the presence of yoghurt cultures, since at no moment did colonies of *S. salivarius* ssp. *thermophilus or L. delbrueckii* ssp. *bulgaricus* appear, and it was possible to count the viable cells of *B. lactis* even when this number was several logarithmic cycles lower than that of the other cultures present. In Lee's agar *B. lactis* failed to grow under the conditions used, and *S. salivarius* ssp. *thermophilus and L. delbrueckii* ssp. *bulgaricus* were distinguished from each

other by a difference in the colour of the colonies, the former forming bright yellow colonies and the latter cream coloured colonies, as can be seen in Figure 5.

According to DAVE & SHAH (1996), some media containing antibiotics, inhibit the growth of bifidobacterias, the count not therefore being representative of the true number of viable cells present in the product analysed. However, comparing Tables 5 and 6, it can be seen that the supplemented MRS medium, which contained antibiotic, allowed for the counting of, in general, one more cycle than in RCPB, for all cultures at all times.

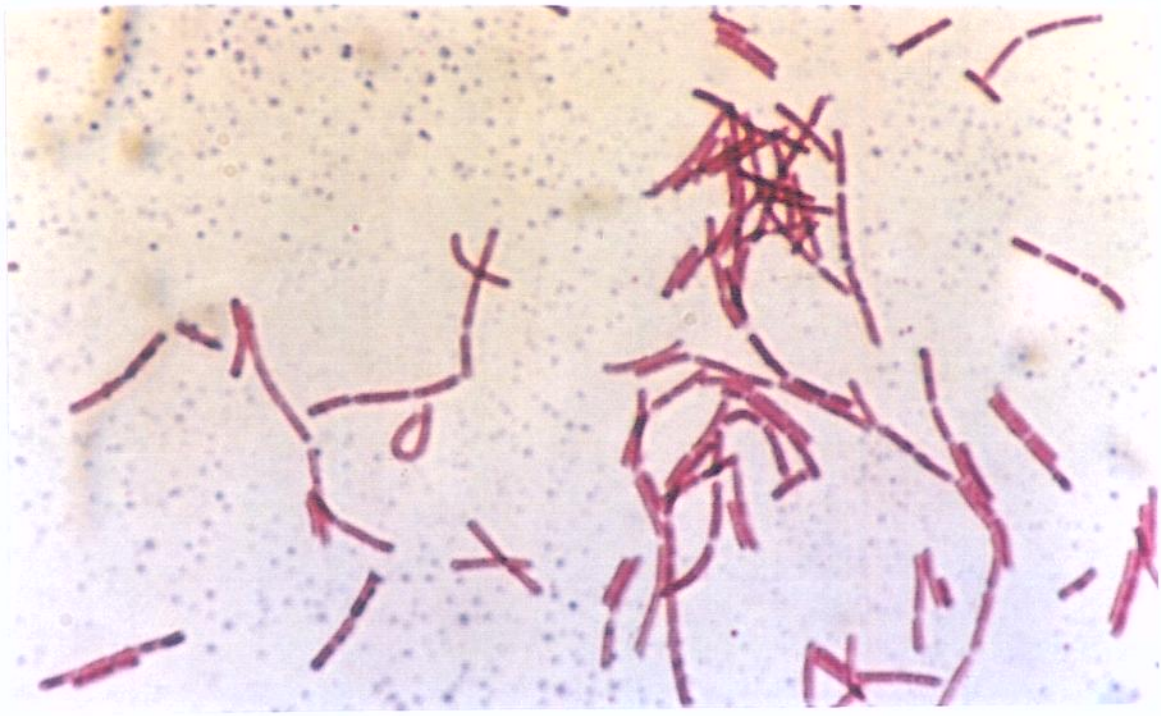

Figure 1. Morphology of *Lactobacillus acidophilus* cells cultivated in MRS medium for 72 hours. Objective ×100.

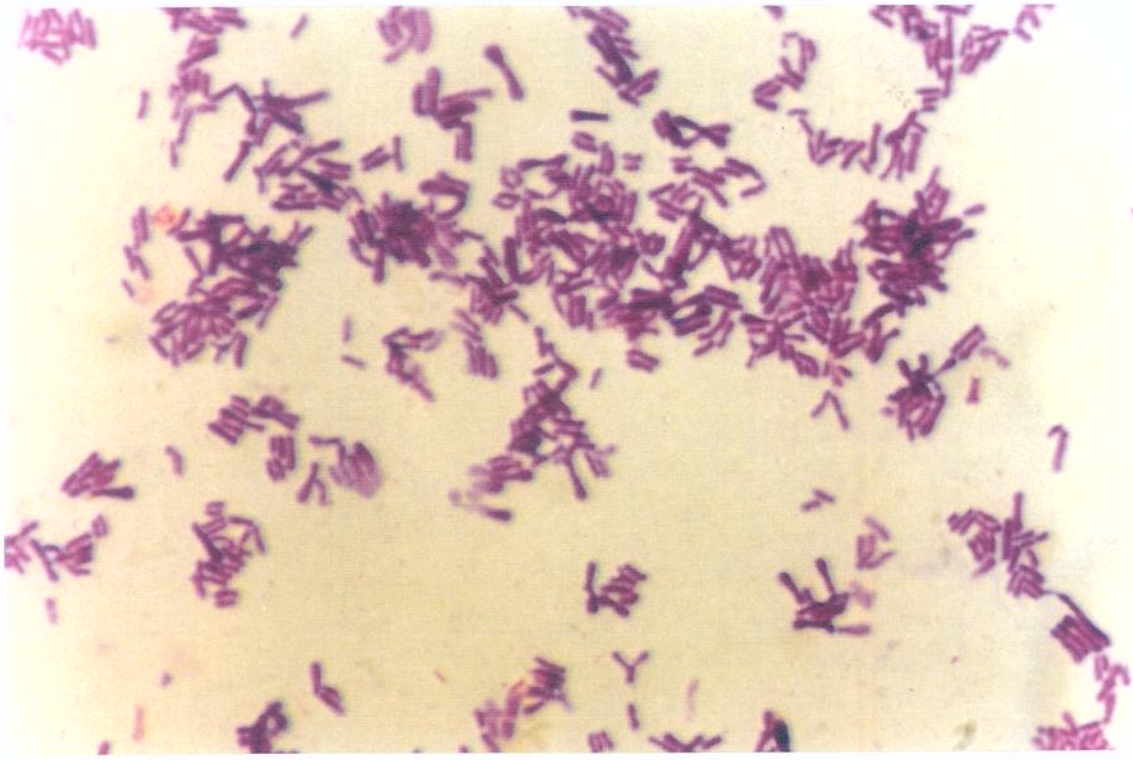

Figure 2. Morphology of *Bifidobacterium lactis* cells cultivated in RCPB medium for 72 hours. Objective ×100.

Figure 3. Morphology of the colonies of *Streptococcus salivarius* ssp. *thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Bifidobacterium. lactis* in RCPB medium, after 48 hours of incubation.

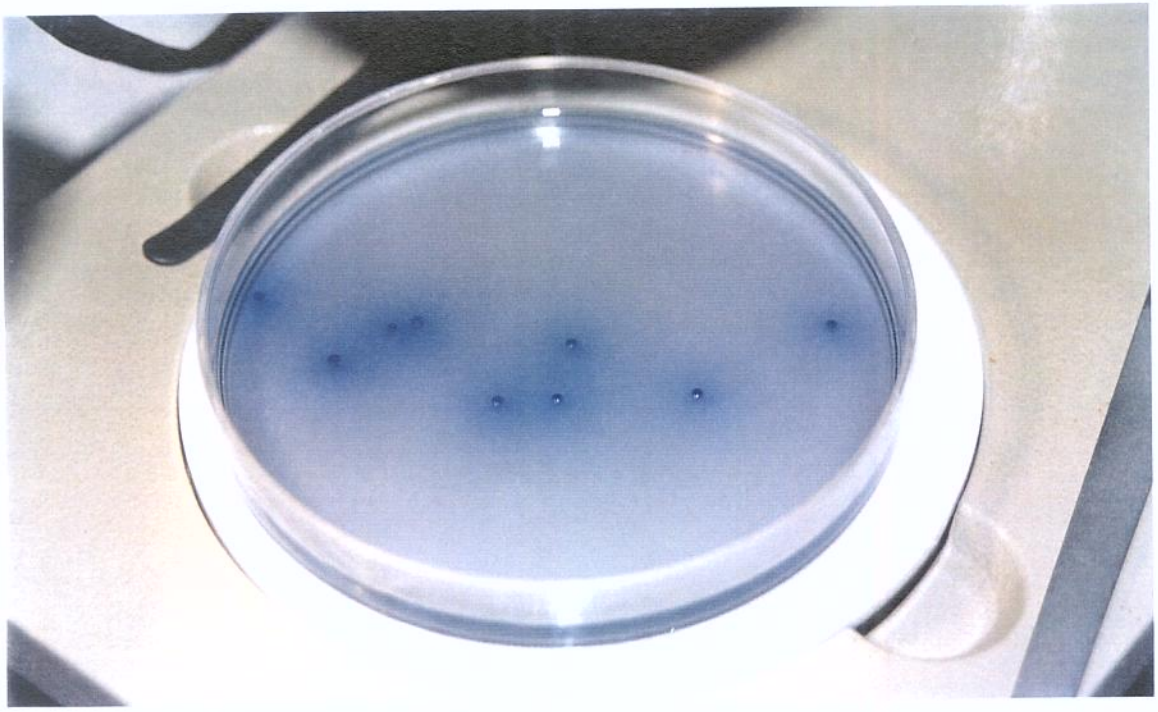

Figure 4. Morphology exhibited by the colonies of *Bifidobacterium lactis* in the supplemented MRS medium, after 72 hours of incubation.

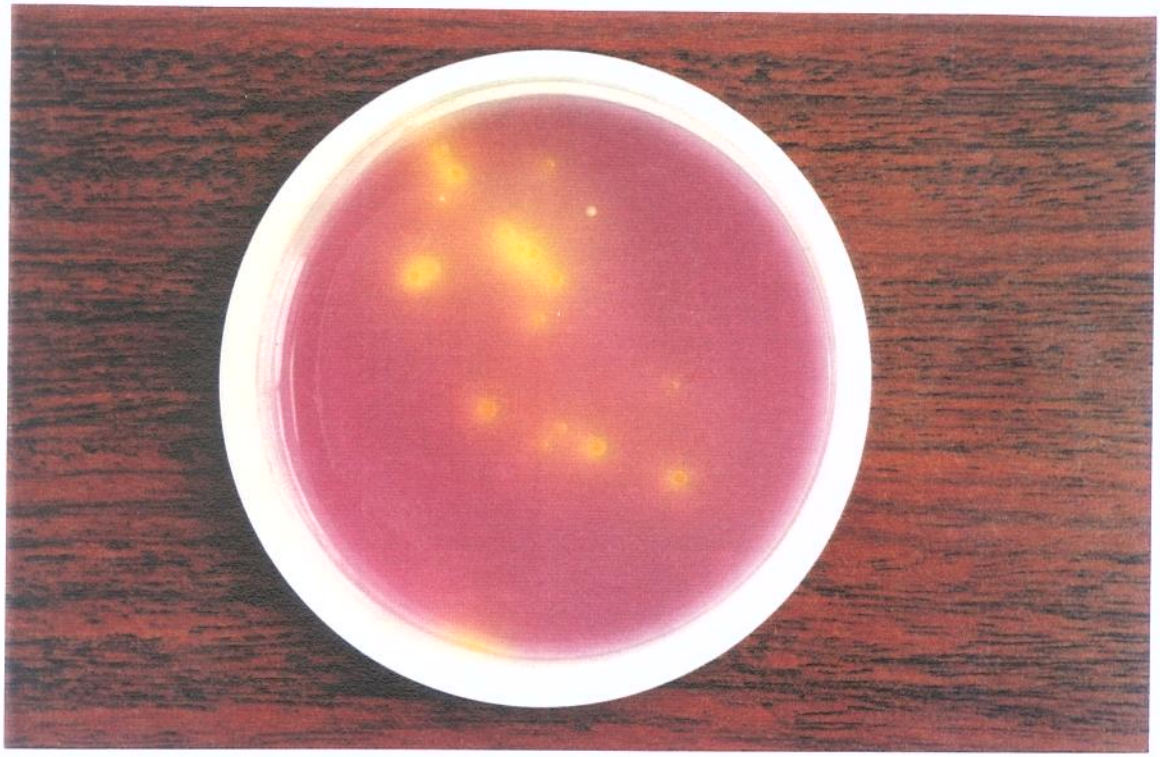

Figure 5. Morphology of the colonies of *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in Lee's agar after 48 hours of incubation.

Evaluation of the stability of *L. acidophilus* and of *B. lactis* in their free and immobilised forms in acidified milk.

Free and immobilised *L. acidophilus* showed good stability in acidified milk, the viable count being reduced by only one logarithmic cycle after 14 days storage at pH values of 4.4 and 3.8 (Table 1 and 2). This result is in agreement with that reported by LAROIA & MARTIN (1991), in which *L. acidophilus* survived in great numbers in a frozen fermented product with pH values varying from 3.9 to 4.6, and with that reported by GILLILAND & SPECK (1977), where *L. acidophilus* remained viable in milk acidified with lactic acid.

The survival of free *B. lactis* in acidified milks was considered satisfactory, since a reduction of only one logarithmic cycle was registered after between 21 and 28 days of storage at an initial pH of 5.0, and after between 7 and 14 days at

pH 4.4. Milk at pH 3.8 most influenced the survival of *B. lactis,* showing a reduction of one logarithmic cycle after only 7 days storage and of another one after 21 days (Table 3). Immobilised and free *B. lactis* showed very similar performance. However at pH value 3.8 the immobilised cells suffered a reduction of only 1 logarithmic cycle (Table 4). This result differs from that of LAROIA & MARTIN (1991), in which *B. lactis* failed to survive in a product with a pH varying from 3.9 to 4.6. However, according to LANKAPUTHRA *et al.* (1996), the resistance during refrigerated storage in acid products, varies between the different species of bifidobacterias. These researchers tested the viability of 9 species of bifidobacteria in acidified milk at pH values of 4.3, 4.1, 3.9 and 3.7, stored under refrigeration for 42 days, and showed that only three species, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099, were capable of surviving in good numbers, the remaining species (*B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210), were exterminated by the low pH or presence of H_2O_2.

Table 1. Stability of free *L. acidophilus* incorporated into milk (pH 6.4) and into acidified milk at pH values of 5.0; 4.4 and 3.8 (cfu/ml).

	Initial pH			
Time (days)	6.4	5.0	4.4	3.8
0	9.8×10^{7a}	3.1×10^{7a}	3.1×10^{7a}	8.2×10^{7a}
7	8.3×10^{7a}	3.8×10^{7a}	3.4×10^{7a}	6.0×10^{7a}
14	4.7×10^{7a}	3.5×10^{7a}	6.1×10^{7a}	7.3×10^{7a}
21	1.1×10^{7a}	2.4×10^{7a}	1.1×10^{6b}	3.0×10^{6b}
28	1.0×10^{7a}	2.0×10^{7a}	3.5×10^{6b}	1.6×10^{6b}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Table 2. Stability of immobilised *L. acidophilus* incorporated into milk (pH 6.4) and into acidified milk at pH values of 5.0; 4.4 and 3.8 (cfu/ml).

	Initial pH			
Time (days)	6.4	5.0	4.4	3.8
0	1.3×10^{7a}	1.8×10^{7a}	8.4×10^{6a}	7.5×10^{6a}
7	1.1×10^{7a}	1.1×10^{7a}	8.9×10^{6a}	6.1×10^{6a}
14	6.2×10^{7a}	6.4×10^{7a}	6.7×10^{6a}	2.4×10^{6a}
21	1.5×10^{6b}	2.5×10^{6b}	1.3×10^{5b}	3.8×10^{5b}
28	1.8×10^{6b}	1.1×10^{6b}	4.5×10^{5b}	1.2×10^{5b}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Table 3. Stability of free *B. lactis* in milk (pH 6.4) and in milk acidified to pH values of 5.0; 4.4 and 3.8 (cfu/ml).

	Initial pH			
Time (days)	6.4	5.0	4.4	3.8
0	8.4×10^{7a}	5.4×10^{7a}	3.2×10^{7a}	8.9×10^{7a}
7	7.0×10^{7a}	5.6×10^{7a}	6.0×10^{7a}	9.0×10^{7a}
14	7.7×10^{7a}	4.1×10^{7a}	4.4×10^{6b}	6.3×10^{6b}
21	3.6×10^{7a}	3.5×10^{7a}	9.0×10^{6b}	3.2×10^{6b}
28	2.0×10^{7a}	2.7×10^{6b}	2.7×10^{6b}	1.0×10^{5c}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Table 4. Stability of immobilised *B. lactis* in milk (pH 6.4) and in milk acidified to pH values of 5.0; 4.4 and 3.8 (cfu/ml).

	Initial pH			
Time (days)	6.4	5.0	4.4	3.8
0	2.5×10^{7a}	3.5×10^{7a}	2.4×10^{7a}	1.1×10^{7a}
7	1.5×10^{7a}	3.1×10^{7a}	1.9×10^{7a}	1.1×10^{7a}
14	1.6×10^{7a}	3.3×10^{7a}	5.9×10^{7a}	4.2×10^{6b}
21	4.0×10^{7a}	5.2×10^{6b}	1.9×10^{6b}	2.4×10^{6b}
28	9.9×10^{6b}	3.2×10^{6b}	2.9×10^{6b}	2.0×10^{6b}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Evaluation of the stability of immobilised *B. lactis* in yoghurt

One of the requirements for microorganisms to be used for therapeutic purposes is that they remain viable in the food used as a vehicle for their consumption (KIM, 1988). *B. lactis* immobilised in calcium alginate was incorporated into high acid yoghurt (pH 4.2) and a gradual decline in the number of viable cells was shown throughout the storage period (table 5).

According to LAROIA & MARTIN (1991) and to MARTIN & CHOU (1992), the low pH of fermented products is effectively malefic to some species/strains of bifidobacterias. However it was shown that free and immobilised *B. lactis* incorporated into acidified milks (pH 4.4 and 3.8) presented a much higher survival rate than in yoghurt at similar pH values, since they only suffered a maximum reduction in viable count of two logarithmic cycles after 28 days of storage (Tables 3 and 4), whilst in yoghurt the whole population was exterminated in the same period (Table 5). Thus the survival of the free and immobilised *B. lactis* in yoghurt, must have been affected by other factors, such as inhibitory substances produced by the yoghurt culture or an excess of dissolved oxygen. The alginate matrix failed to function as a barrier to these factors. This results conflicts with that of KHALIL & MANSOUR (1998) in which immobilisation was effective in the protection of *Bifidobacterium bifidum* and *B. infantis* cells immobilised in alginate and incorporated into mayonnaise. Immobilisation in alginate also improved the survival of *Lactobacillus bulgaricus* in a milk based dessert (SHEU *et al.*, 1993).

The populations of *L. delbrueckii* ssp. *bulgaricus* and *S. salivarus* ssp. *thermophilus* suffered reductions of two and one logarithmic cycles, respectively, during the experiment (Tables 5 and 6). This result confirms the domination of *S. salivarus* ssp. *thermophilus* during the refrigerated storage of yoghurt prepared with addition of *B. lactis*, as reported by RYBKA & KAILASPATHY (1995).

Table 5. Counts of immobilised *B. lactis* (in supplemented MRS medium), *Streptococcus salivarus* ssp. *thermophilus* & *Lactobacillus delbrueckii* ssp. *bulgaricus* (in Lee's agar) in yoghurt at pH 4.2, during 28 days of storage, at 7°C (cfu/ml).

Storage (days)	*B. lactis*	*S. thermophilus*	*L. bulgaricus*
0	7.2×10^8	8.1×10^8	9.5×10^8
7	8.6×10^6	5.0×10^8	6.7×10^8
14	1.9×10^5	6.9×10^8	4.6×10^7
21	5.4×10^3	8.4×10^7	3.0×10^7
28	-	5.1×10^7	1.8×10^6

- There was no count at the lowest dilution plated (10^{-1}).

Table 6. Count of immobilised *B. lactis*. *Streptococcus salivarus* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (all in RCPB medium) in yoghurt at pH 4.2, during 28 days of storage at 7°C (cfu/ml).

Storage (days)	*B. lactis*	*S. thermophilus*	*L. bulgaricus*
0	6.0×10^7	9.1×10^7	8.0×10^7
7	2.9×10^6	3.4×10^7	6.3×10^7
14	-	6.0×10^7	5.9×10^7
21	-	9.6×10^6	1.1×10^7
28	-	1.8×10^6	8.7×10^5

- No count was possible.

Conclusions

Free and immobilised *B. lactis* and *L. acidophilus* presented a good survival rate in acidified milk. The survival of immobilised *B. lactis* in yoghurt was considered to be unsatisfactory, that is, immobilisation in calcium alginate failed to produce an effective barrier to protect the cells.

Both RCPB and supplemented MRS were efficient media for counting *B. lactis* in yoghurt whilst the cultures were in equilibrium; when *L. delbrueckii* ssp.

bulgaricus and *S. salivarus* ssp. *thermophilus* prevailed, the differential medium did not allow for the counting of *B. lactis,* the selective medium being more efficient.

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MICROENCAPSULATION OF *L. acidophilus* AND *B. lactis* BY SPRAY DRYING

Trabalho enviado ao periódico
Journal of Dairy Science

ABSTRACT

The objective of this study was to prepare microcapsules with symbiotic properties using the probiotic microorganisms *Lactobacillus acidophilus* and *Bifidobacterium lactis,* a oligofructose with prebiotic properties, the spray drying technique and cellulose acetate phthalate as the wall material. The resistance of these organisms to drying at three distinct temperature and the survival rate during storage at 25°C for 90 days were determined. The morphology of the microcapsules was determined by scanning electron microscopy. The average size and distribution was also determined. The microcapsules presented a cylindrical shape, continuous wall with no apparent porosity, average size of 22 μm, moisture content varying from 5.3 to 3.2% and water activity between 0.230 and 0.204. The higher the spray drying temperature, the lower the survival rate. With an air entry temperature of 130°C and exit temperature of 75°C, the number of viable cells of *B. lactis* was practically unaltered, whereas the population of *L. acidophilus* was reduced by 2 logarithmic cycles. *B. lactis* was more resistant to the drying process than *L. acidophilus* under all conditions tested, and was also more resistant during storage, suffering a reduction of 2 logarithmic cycles after 60 days, whereas the population of *L. acidophilus* was reduced by 5 logarithmic cycles after 90 days of storage.

(**Key words**: probiotic, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, microencapsulation by spray-drying)

INTRODUCTION

Probiotic cultures have been used in foods as an adjunct due to the beneficial effects attributed to them with respect to human health (16). However, in order to exert a beneficial effect on the host, they must be viable in the food at the moment of ingestion in numbers of approximately 10^6 cfu/ml (5). The survival of these cultures in foods is open to question and has given rise to much controversy, since some strains are extremely sensitive, and intolerant of acids, oxygen, bile and the presence of other cultures. In addition, these cultures normally contribute little to the sensory quality of the product (11).

Prebiotics are non digestible food ingredients with a beneficial effect for the consumer since they select, stimulate the growth and activate the metabolism of probiotic bacteria in the intestine (9). Symbiotic products contain a mixture of prebiotics and probiotics, which, by synergism, will benefit the host (8).

Microencapsulation is a process by which particles are formed containing an active ingredient covered by a fine layer of another material, which provides protection and controlled liberation as well as convenience to the ingredient. It is the composition of this wall material which mostly determines the functional properties of the microcapsules (10). Cellulose acetate phthalate is one of the wall materials which acts as a barrier to acid pH values, and is also a physiologically inert polymer when administered *in vivo*, being widely used as a wall material in capsules for the liberation of drugs in the intestine, since it is insoluble in acid mediums, but soluble at pH values equal or greater than 6.0 (13). Despite this,

there are few reports of the use of this polymer as a wall material in the preparation of microcapsules by spray drying.

A good survival rate for *Pseudomonas fluorescens-putida* was reported after one year of storage when microencapsulated by spray drying with the polymer Eudragit® (1). However, the survival rate of spray dried *L. acidophilus* was considered unsatisfactory after 30 days of storage (6).

The objectives of this study were to elaborate microcapsules with symbiotic properties using the probiotic microorganisms *L. acidophilus* and *B. lactis*, a oligofructose as prebiotic, cellulose acetate phthalate as wall material and spray drying as the method of preparation, verifying the effect of different drying temperatures on the microorganisms, and characterising the microcapsules microscopically and by an evaluation of the particle size and distribution. The survival rate during storage at 25°C for 90 days was also determined.

MATERIALS AND METHODS

Materials

Cellulose acetate phthalate (Eastman Kodak Co., Rochester, NY), maltodextrin (Corn Products International, Mogi Guaçu, Brazil), raftilose® (Orafti, Tienen, Belgium), tween 80 (Synth, São Paulo, Brazil) and glycerol (Synth, São Paulo, Brazil).

Cultures

Lactobacillus acidophilus (La-05) and *Bifidobacterium lactis* (Bb-12) (Chr. Hansen, Valinhos, Brazil), in the DVS (direct vase set) form, pure and freeze dried, were maintained in the proportion of 1g per 150 ml of a sterile solution of 12% reconstituted skimmed milk, at a temperature of -18°C.

Preparation of Microcapsules

The microcapsules were obtained by spray drying the formulation presented in Table 1, in a spray dryer model SD 04 (Lab-Plant, Huddersfield, UK), using the operating conditions stipulated in Table 2. Each treatment was processed in triplicate, using collection flasks, tubing, spatulas and caps all sterilised at 121°C for 15 minutes. The formulation used was determined in preliminary tests. Maltodextrin and raftilose® were added with the objective of increasing the density of the powder and producing microcapsules with symbiotic properties, respectively.

Table 1. Formulation used for the elaboration of microcapsules of *B. lactis* and *L. acidophilus*.

Material	Function	Quantity
Phosphate buffer pH 8 (ml)	Solvent	100.0
Cellulose acetate phthalate (g)	Wall material	10.0
Glycerol (g)	Plasticizer	3.5
Maltodextrin (g)	Adjustment of density	2.0
Raftilose® (g)	Prebiotic	1.0
Tween 80 (g)	Surfactant	0.1
Reconstituted milk (ml)	Carrier of the microorganisms	10.0

Table 2. Operating conditions of the *spray-dryer* in the microencapsulation processes.

Parameters	Conditions
Temperature at entrance (°C)	130, 160, 190
Mean temperature at exit (°C)	75, 100, 120
Air pressure (kgf/cm^2)	5
Flow rate of peristaltic pump (ml/min)	5
Nozzle diameter (mm)	0.5

Microbial Count

Reinforced Clostridial Agar (RCA) (Oxoid, Hampshire, UK) plus 0.01% aniline blue (Nuclear, São Paulo, Brazil) was used to enumerate *B. lactis. L. acidophilus* was enumerated according to Fávaro-Trindade & Grosso (7). Both cultures were plated by the pour plate technique. A 2% solution of sodium citrate (Synth, São Paulo, Brazil) was used to prepare the serial dilutions. The plates were incubated in jars containing Anaerobac (Probac, São Paulo, Brazil) anaerobiosis generating systems, at 37°C for 72 h.

To make it possible to quantify the numbers of viable cells in the microcapsules, it was necessary to dissolve the wall material to liberate the microorganisms. This was done by blending the micro-capsules with 2% sterile sodium citrate solution for 4 minutes at high speed, in a Stomacher 400 homogeniser (Seward, UK).

Determination of the Effect of Drying Temperature on the Survival of *L. acidophilus* and *B. lactis*

Counts were made in the spray dryer feed solutions and in the microcapsules one hour before and one hour after processing, respectively, with the objective of determining the resistance of *B. lactis* and *L. acidophilus* at the different temperatures used in the process to obtain the microcapsules.

Analysis of the Stability of the Microencapsulated Organisms

The quantities of viable cells of *B. lactis* and *L. acidophilus* in the microcapsules was monitored by counts in the powders after 30, 60 and 90 days of storage at 25°C in capped glass containers.

Moisture Content and Water Activity

The moisture content was determined in triplicate using the methodology of A.O.A.C. (2). Water activity was determined in triplicate using the AQUALAB equipment (Decagon Devices, Pullman, WA).

Analysis of Particle Size and Distribution

The particle size and distribution was evaluated using the Lumosed Photo-Sedimentometer of Anton Paar (model A-8054, Retsch, Haan, Germany) with a sedimentation medium of isobutanol (Synth, São Paulo, Brazil). Sample quantities of 30 mg were used and an analysis time of 40 min. The determinations were made in triplicate.

Scanning Electron Microscopy

The morphology of the microcapsules was observed by scanning electron microscopy. The encapsulated samples were fixed in stubs on a double faced metallic tape and covered with a fine layer of gold using a Balzers evaporator (model SCD 050, Baltec, Lichteinstein, Austria) for 120 s, applying a current of 40

mA. Observations were made using the Scanning Electron Microscope (JEOL, JSM-T300, Tokyo, Japan) at an accelerating voltage of 20 kV (15).

Statistical Analysis

A means difference analysis was used to check for the existence of significant differences between the values obtained, according to Tukey's Test, with the help of the software STATISTICA 6.0 (Microsoft®).

RESULTS

Microcapsule Morphology and Size Distribution

The morphological analysis of the microcapsules (Figure 1) showed that they were spherical, presenting indentations, smooth walled, continuous, with no apparent porosity and a great variety of sizes. Whilst in polymeric matrices of calcium alginate, the distribution of microorganisms retained in the gel was clearly visible (7), this was not possible in cellulose acetate phthalate microcapsules.

The average size of the particles was 22 μm with a variation from 5 to 50 μm. Drying temperature showed no significant effect ($P < 0.05$) on mean particle size, which were 20.0; 23.1 and 22.6 μm (standard deviation of 2.0; 1.2 and 0.8 μm) at temperatures of 130, 160 and 190°C, respectively.

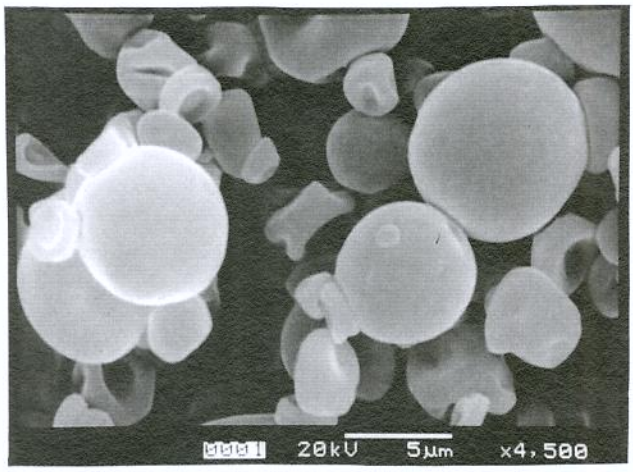

Figure 1. Micrograph of microcapsules containing *L. acidophilus* or *B. lactis* (magnification X4500).

Effect of Drying Temperature on the Survival of *L. acidophilus* and *B. lactis*

During vacuum drying and freeze drying processes, some cells undergo injury, others die and some are unaffected by the process, injury and death probably being caused by a loss of protein from the cell wall as well as losses of bound water, both of which are extremely important for the maintenance of the structural and functional integrity of biological macromolecules (3).

The lower the temperature the greater the survival of spray dried microencapsulated *B. lactis* and *L. acidophilus*, as can be seen in Table 3. This same effect was also observed for *L. acidophilus* (6), for *Salmonella* (12) and for *L. bulgaricus* (17). The lowest entrance/exit temperature was the least lethal for the

microorganisms, the population of *B. lactis* showing practically no alteration, going from $1.75x10^8$ cfu/g at the start of the process to $1.32x10^8$ cfu/g at the end. This result was similar to that obtained for *L. bulgaricus* at an entrance temperature of 200°C and exit temperature of 80°C (18). The population of *L. acidophilus* suffered a reduction of 2 logarithmic cycles with drying temperatures of 130/75°C. A similar result was obtained by Espina & Packard (6) who reported a loss of at least two logarithmic cycles when they dried *L. acidophilus* in a spray dryer, using an entry temperature of 170°C and exit temperatures of 75, 80 and 85°C.

B. lactis was shown to be more resistant to the process than *L. acidophilus* in all the treatments tested, although at the highest drying temperature (190°C/120°C, entrance/exit) its population was drastically reduced by 3 logarithmic cycles.

Moisture content and water activity also decreased with increases in drying temperature. The values determined for moisture content and water activity were respectively 5.3% and 0.230; 3.8% and 0.226 and 3.2% and 0.204 for the temperatures of 130, 160 and 190°C, respectively.

Table 3. Survival of *L. acidophilus* and *B. lactis* during the spray drying process.

Microorganism	Drying temperature (°C)		(cfu/g of solids)	
	Entrance	Mean exit	Before the process	After the process
B. lactis	130	75	$1.75x10^8$	$1.32x10^8$
	160	100	$1.00x10^8$	$2.46x10^7$
	190	120	$3.45x10^8$	$7.85x10^5$
L. acidophilus	130	75	$1.20x10^8$	$2.32x10^6$
	160	100	$5.71x10^8$	$1.50x10^6$
	190	120	$1.20x10^8$	$1.86x10^4$

Stability of the Microencapsulated Microorganisms During Storage

Microcapsules obtained at 130/75°C were used for the storage study, since *B. lactis* and *L. acidophilus* showed their greatest survival rate under these conditions.

Figure 2 shows the stability of microencapsulated *B. lactis* and *L. acidophilus* during a storage period of 90 days. It can be seen that the number of viable cells of *B. lactis* remained unaltered during the first month of storage, but subsequently suffered a reduction of 2 logarithmic cycles from 30 to 60 days of storage, then tending to remain unaltered up to the end of the experiment. The number of viable cells of *L. acidophilus* suffered a great reduction (4 logarithmic cycles) during the first month of storage, and the reduction tended to continue thereafter. Prajapati *et al* (14) reported the same tendency when *L. acidophilus* was spray dried mixed with various different products, although in a mixture with banana puree and tomato juice, the population of *L. acidophilus* suffered only a slight reduction during a storage period of 60 days at room temperature. A reduction of 50% of the population of spray dried *L. acidophilus* after 30 days storage has previously been reported (6). According to Teixeira *et al.* (19), even if the cells survived the drying operation, it is possible that they suffer injury during the process, which could result in considerable losses during the subsequent storage period.

Pseudomonas, microencapsulated in modified starch, did not survive even a week, since this polymer has a high water affinity and could have absorbed the intracellular fluid of these bacteria, which would be lethal (1). The same authors

reported the necessity to maintain the residual moisture content of the powders between 8 and 10% to assure the survival of microencapsulated bacteria.

The storage temperature, strain used and residual water content were considered to be determining factors for the survival of microorganisms subjected to drying processes (4). These authors evaluated the survival and enzymatic activity of freeze dried *B. longum* during storage and observed a considerably lower loss in enzymatic activity as compared to the decrease in viable cells, thus concluding that non-viable cells could still express biological functions.

Thus the low residual moisture content after drying (5.35%) and the relatively high storage temperature (25°C) could have negatively influenced the survival of microencapsulated *B. lactis* and *L. acidophilus* during the storage experiment.

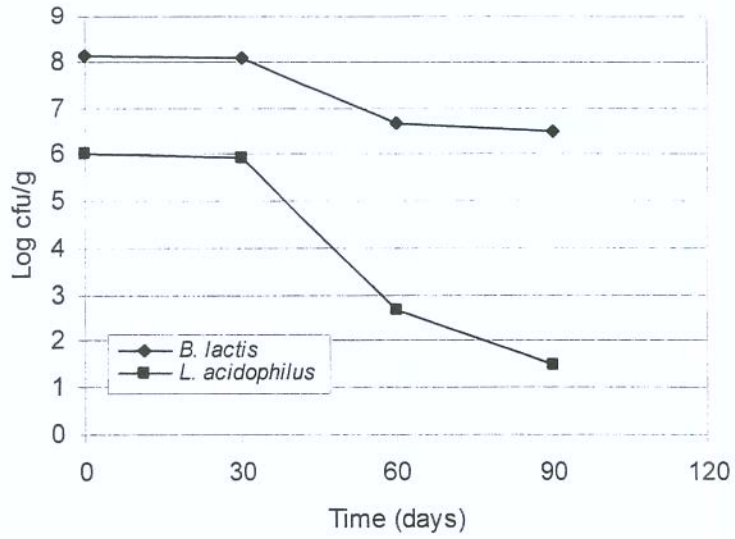

Figure 2. Stability of *L. acidophilus* and *B. lactis* microencapsulated at a drying temperature of 130/75°C and stored at a temperature of 25°C for 90 days.

CONCLUSIONS

The survival of spray dried microencapsulated *B. lactis* and *L. acidophilus* was shown to be dependent on the drying temperature used, since lower temperatures resulted in higher survival rates. *B. lactis* was more resistant than *L. acidophilus* under all the conditions tested, and also during storage. For both microorganisms, survival during storage was only considered satisfactory during the first month.

Microencapsulation by spray drying using the enteric polymer cellulose acetate phthalate as wall material, was shown to be a convenient process to obtain microcapsules with symbiotic properties, especially when using *B. lactis*. Although survival was observed, the process conditions could be optimised, and it is also necessary to evaluate if microencapsulated *B. lactis* and *L. acidophilus* are capable of surviving undamaged during their passage through the gastrointestinal system.

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SURVIVAL OF MICROENCAPSULATED *Lactobacillus acidophilus AND Bifidobacterium lactis* AT THE pH VALUES OF THE STOMACH, IN BILE AND IN YOGHURT

Trabalho enviado ao periódico
Lebensmittel Wissenschaft Technologie

Abstract

This study evaluated the "in vitro" tolerance of microencapsulated *Bifidobacterium lactis* and *Lactobacillus acidophilus* to pH values and bile concentrations similar to those found in the human stomach and intestine, respectively, and also the stability of *B. lactis* in yoghurt, both in the free form and microencapsulated in cellulose acetate phthalate. The free and microencapsulated microorganisms were inoculated in the proportion of 10%, into HCl solutions with their pH values adjusted to 1 and 2, and then incubated anaerobically at 37°C, plating after 0, 1 and 2 h of incubation. The bile concentrations tested were 0, 2 and 4% and the counts were carried out after periods of 0 and 12 h of incubation under anaerobic conditions. The viability of free and microencapsulated *B. lactis* was determined during 28 d of refrigerated storage after addition to yoghurt previously fermented to pH 4.2. Morphological observations were made using scanning electron microscopy. Microencapsulated *B. lactis* and *L. acidophilus* were resistant to conditions similar to those of the stomach. However they were not more resistant than the free cells when added to yoghurt. The process of microencapsulation did not affect the resistance of *B. lactis* and *L. acidophilus* to the presence of bile.

Keywords: probiotics; *Lactobacillus acidophilus; Bifidobacterium lactis;* microencapsulation by spray drying; cellulose acetate phthalate.

Introduction

Many studies have reported on the therapeutic effects of probiotic bacteria such as *L. acidophilus* and the bifidobacteria (1, 2, 3, 4, 5, 6). However, in order to exert these beneficial effects on the host, these organisms must remain viable in the food up to the moment of consumption, in numbers of at least 10^6 cfu/ml and must be capable of surviving during their passage through the gastrointestinal tract (7). The resistance to acid pH values and to the presence of bile can vary considerably between different strains of the same species, both for *Lactobacillus acidophilus* and for *Bifidobacterium* spp. (8).

Microencapsulation techniques and the immobilisation of microorganisms in gels, have been applied, with the objective of providing protection to the bacteria (9, 10, 11, 12, 13, 14, 15, 16).

L. acidophilus and *B. lactis* immobilised in calcium alginate were not more tolerant of acid pH values than the free cultures (17). However, the microencapsulation of *B. pseudolongum* in cellulose acetate phthalate, using coacervation with phase separation, provided a greater survival of this culture during incubation in gastric and intestinal juices (9).

L. acidophilus and *Bifidobacterium* spp. do not always survive in yoghurt in satisfactory numbers, due, amongst other factors, to the acid intolerance of some strains and the presence of other cultures such as *L. delbrueckii* subsp. *bulgaricus* (18, 19). Nevertheless, alginate encapsulated *B. bifidum* and *B. infantis* survived various weeks in mayonnaise (pH 4.42) whilst the free cells were completely destroyed in one week (15).

The objectives of this study were to evaluate the tolerance of *Bifidobacterium lactis* and *Lactobacillus acidophilus*, microencapsulated in cellulose acetate phthalate, by spray drying, to pH values and bile concentrations similar to those found in the human stomach and intestine respectively, and determine the survival of free and microencapsulated *B. lactis* when added to yoghurt.

Materials and Methods

Cultures

Lactobacillus acidophilus (La-05), *Bifidobacterium lactis* (Bb-12) and a mixed culture of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Chr. Hansen, Valinhos, Brazil), in the DVS (direct vase set) form, pure and freeze dried, were maintained in the proportion of 1 g per 150 ml of a sterile solution of 12% reconstituted skimmed milk, at a temperature of -18°C. The mixed culture was replicated twice before use, incubating 2% of the original inoculum in 12% sterile skimmed milk at 45°C for 3 h.

Culture counts

L. acidophilus was enumerated according to Fávaro-Trindade & Grosso (17). Reinforced Clostridial Agar (RCA) (Oxoid, Hampshire, UK) plus 0.01% aniline blue (Nuclear, São Paulo, Brazil) was used to enumerate *B. lactis* in the absence of other cultures, by the pour plate technique.

In the yoghurt, *B. lactis* was enumerated in MRS broth (Oxoid, Hampshire, UK) supplemented with: 0.5% of a 10% solution of L-cysteine hydrochloride (Synth,

São Paulo, Brazil), 0.5% of a solution of dicloxacillin (10 mg/100 ml water) (Sigma, Louis, Mo, USA) and 1% of a 10% solution of lithium chloride (Vetec, Brazil) (35, 36), modified by the addition of 0.01% aniline blue (Nuclear, Br) by the spread plate technique. This medium was used since it permits the growth of *B. lactis* but inhibits the growth of *St. thermophilus* and *B. bulgaricus.*

St. salivarius subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were plated in Lee's agar by the spread plate technique, and incubated for 48 h at 37°C in jars containing a microaerophilic generating system, Microaerobac (Probac, São Paulo, Brazil). Under these conditions there is no growth of *B. lactis,* and *St. Salivarius* subsp. *thermophilus* grows forming bright yellow colonies whilst *L. delbrueckii* subsp. *bulgaricus* forms cream coloured colonies.

Under all the above cited conditions, *B. lactis* and *L. acidophilus* were incubated for 72 h at 37°C in jars containing an anaerobic generating system, Anaerobac (Probac, São Paulo, Brazil), 2% sodium citrate being used as diluent and the platings being effected in duplicate.

For the quantitative measurements of the viable cells, it was necessary to solubilize the microcapsules to liberate the microorganisms. This was effected by blending for 4 min at high speed in 2% sterile sodium citrate at room temperature, using a Stomacher 400 homogeniser (Seward, London, UK).

Preparation of microcapsules

In this study the microcapsules were prepared by drying in a spray dryer (Lab-Plant, SD-04, Huddersfield, UK) at temperatures of 130°C/75°C (entrance and exit

air temperatures, respectively), using a formulation containing cellulose acetate phthalate (Eastman Kodak Co., Rochester, NY), maltodextrin (Corn Products International, Mogi Guaçu, Brazil), raftilose® (Orafti, Tienen, Belgium), tween 80 (Synth, São Paulo, Brazil), glycerol (Synth, São Paulo, Brazil) and *L. acidophilus* or *B. lactis*.

Yoghurt preparation

The yoghurt was prepared using whole powdered milk, reconstituted at 15% solids and sterilised at 121°C for 10 min. Fermentation was carried out with a 2% inoculum (mixed culture of *St. Salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus,* previously replicated in milk), incubating at 45°C until a pH value of 4.2. The yoghurt was homogenised for 5 min. The homogenised product was distributed into plastic cups and 1.5% of either free (dissolved in milk) or microencapsulated *B. lactis* added. The contents were lightly homogenised using a spatula, the cups capped and stored in a BOD incubator (Tecnal model TE 390, Piracicaba, Brazil) for 28 d at 7°C.

Survival of microencapsulated L. acidophilus and B. lactis at pH levels similar to those of the human stomach

The determination of the effect of pH values similar to those found in the human stomach on microencapsulated *L. acidophilus* and *B. bifidum,* was carried out in triplicate according to Clark et al. (20), using distilled water pH 7.2 as control.

Survival of microencapsulated L. acidophilus and B. lactis in bile concentrations similar to those of the human intestine.

This test was carried out in triplicate using the methodology of Clark & Martin (21) using Oxgall bile solutions (Difco, Detroit, USA) and peptone water as control.

Stability of free and microencapsulated B. lactis in yoghurt

With the objective of determining the stability of free and microencapsulated *B. lactis* in yoghurt at pH 4.2, counts of this organism and of *St. Salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were effected in duplicate after 0, 7, 14, 21 and 28 d of storage at 7°C.

Morphology of the microcapsules

The morphology of the microcapsules was observed by scanning electron microscopy. The samples were fixed in stubs on a double faced metallic tape and covered with a fine layer of gold using a Balzers evaporator (model SCD 050, Baltec, Lichteinstein, Austria) for 120 s, applying a current of 40 mA. Observations were made using the Scanning Electron Microscope (JEOL, JSM-T300, Tokyo, Japan) at an accelerating voltage of 20 kV (22). The microcapsules inoculated at pH 1 were removed from the solution after 0, 1 and 2 h of incubation, dried at room temperature and fixed in stubs as above.

Statistical analysis

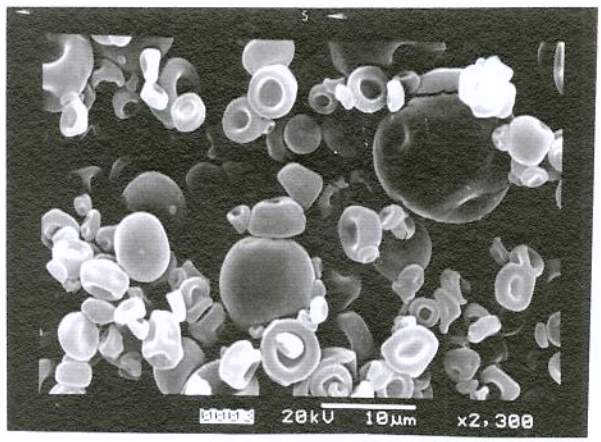
A means difference analysis was used to check for the existence of significant differences between the values obtained, according to Tukey's Test, with the help of the software STATISTICA 6.0 (Microsoft®).

Results and Discussion

Morphology

The morphological analysis of the microcapsules, carried out soon after the microencapsulation procedure (**Fig. 1a**) showed that they were spherical, presenting indentations as a result of the drying process, smooth walled, continuous, without apparent porosity and presenting a great variety of sizes. **Figure 1b** shows that the microcapsules are apparently hollow.

The visualisation of the microcapsules submitted to pH 1, indicated that this material failed to dissolve, the formation of small agglomerates being observed. This agglomeration was confirmed by electron microscopy. Immediately after immersion in the solution at pH 1, agglomerates could be observed, which probably formed as a function of their nearness to each other (**Fig. 2a**), an effect not observed in the microcapsules which did not suffer this treatment at pH 1 (**Fig. 1a** and **2a**). After 1 h the spherical form had been lost, the formation of an agglomerate covered by microcapsules of non-specific geometry being observed (**Fig. 2b**). After 2 h, the agglomeration and deformation suffered by the microcapsules was evident, and the spherical form was completely lost (**Fig. 2c**).



a

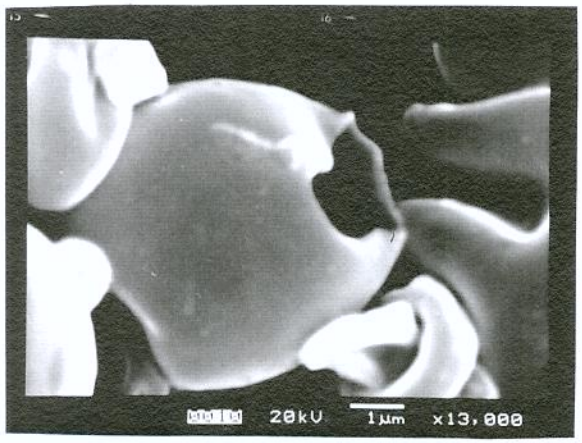


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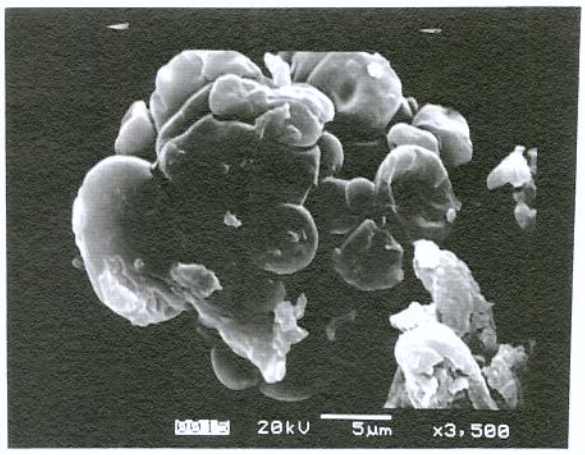
Fig. 1 Micrographs of the morphology of microcapsules:
a. morphology and size distribution; b. detail of the interior of a microcapsule



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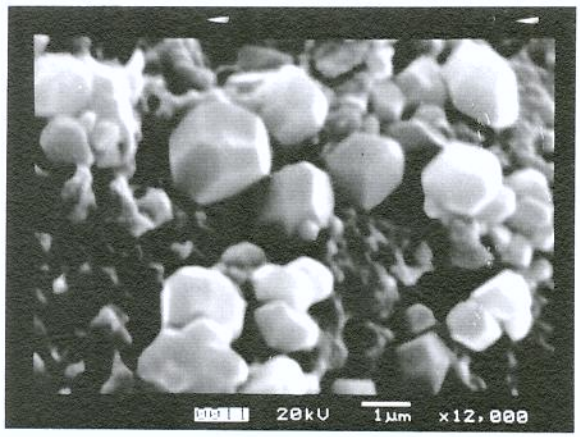

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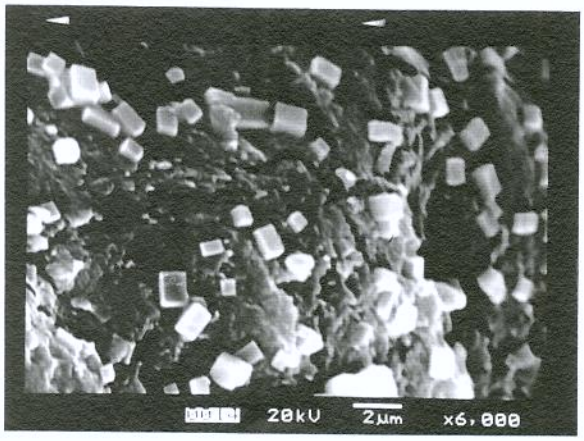

c

Fig. 2 Micrographs of the morphology of microcapsules submitted to immersion in a solution at pH 1: a. zero time; b. after one h; c. after two h

Survival of microencapsulated L. acidophilus and B. lactis at pH values similar to those of the human stomach

As reported in a previous paper (17), free *B. lactis* and *L. acidophilus* underwent a reduction of one logarithmic cycle after inoculation at pH 2 and were completely destroyed after 1 h at pH 1, suggesting that the therapeutic benefits offered by these microorganisms would be somewhat limited if the stomach pH of the host were near to 1. The immobilisation of *B. lactis* and *L. acidophilus* in calcium alginate gel was not effective in protecting the cells submitted to pH values similar to those of the stomach (17), although it was shown to be efficient in the protection of *B. bifidum* and *B. infantis* inoculated into mayonnaise (15), of *Lactobacillus bulgaricus* added to frozen dessert (12) and of *L. bulgaricus* added to ice-cream (11).

Spray dried microcapsules of cellulose acetate phthalate containing *B. lactis* and *L. acidophilus* were efficient in protecting both these microorganisms when inoculated into pH values similar to those of the human stomach (**Figs 3** and **4**). Microencapsulated *L. acidophilus* suffered a reduction of only one logarithmic cycle at pH 1 after 2 h of incubation, and the population of *B. lactis* was reduced by only one logarithmic cycle immediately after inoculation into pH 1, and between 1 and 2 h after inoculation into pH 2. Similar results were obtained by Rao et al. (9), where *Bifidobacterium pseudolongum*, microencapsulated in cellulose acetate phthalate by coacervation survived in greater numbers in the tests with gastric type fluids, than the same microorganism in the free form.

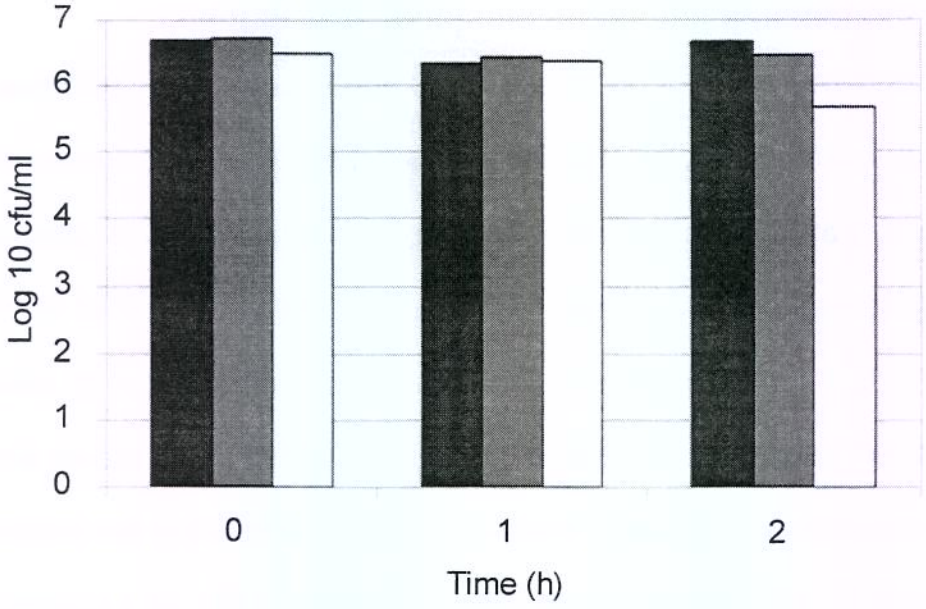

Fig. 3 Effect of pH on the survival of microencapsulated *L. acidophilus.* ■ control; ■ pH 2; □ pH 1

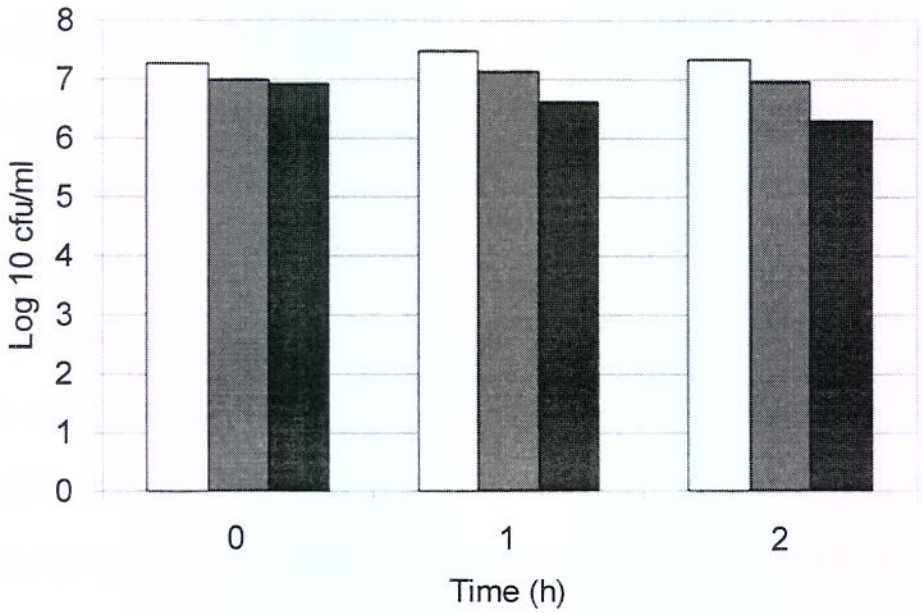

Fig. 4 Effect of pH on the survival of microencapsulated *B. lactis.* □ control; ■ pH 2; ■ pH 1

The survival of microencapsulated L . acidophilus and B. lactis in bile concentrations similar to those of the human intestine

The results presented in **Tables 1** and **2**, referring to the survival of free *L. acidophilus* and *B. lactis* in the presence of bile, confirm the data reported in a previous paper (17), where free *L. acidophilus* and *B. lactis* were shown to be extremely tolerant to bile, even at 4%, which is considered to be greater than the concentrations normally observed in the human intestine. These results are in agreement with those determined by Holcomb et al. (23) but differ from those reported by Clark & Martin (21), where the population of *B. bifidum* was completely destroyed after 12 h incubation in 2% bile or above.

The spray drying process did not alter the tolerance of *B. lactis* and *L. acidophilus* to the presence of bile, which differs from the results reported by Brennan et al. (24), in which the drying of *L. acidophilus* under vacuum and by freeze drying, resulted in intolerance to bile, an effect attributed to losses of specific components from the cell membrane during the drying processes.

After inoculation of the microcapsules into the bile solutions (pH 7), a complete dissolution of the powder was observed, indicating that the wall material and the process used in the preparation of the microcapsules, associated with the survival of the microorganisms, were both adequate with respect to the objective of the microencapsulated microorganisms passing undamaged through the acid conditions of the stomach, followed by their rapid liberation in the basic pH of the intestine.

Table 1 Survival of free and microencapsulated *B. lactis* 12 h after inoculation into bile (cfu/ml)

	Bile concentration (%)		
Condition/Time (h)	0 (control)	2	4
Free / 0	3.1×10^{8Aa}	7.9×10^{8Aa}	6.3×10^{8Aa}
Free / 12	5.6×10^{8Aa}	2.5×10^{8Aa}	4.7×10^{8Aa}
Microencapsulated / 0	2.1×10^{8Aa}	9.1×10^{8Aa}	3.0×10^{8Aa}
Microencapsulated / 12	4.3×10^{8Aa}	3.7×10^{8Aa}	2.4×10^{8Aa}

[A] Means with the same letter in the same line are not significantly different ($P<0.05$)
[a] Means with the same letter in the same column are not significantly different ($P<0.05$)

Table 2 Survival of free and microencapsulated *L. acidophilus* 12 h after inoculation into bile (cfu/ml)

	Bile concentration (%)		
Condition/Time (h)	0 (control)	2	4
Free / 0	3.2×10^{6Aa}	3.4×10^{6Aa}	3.2×10^{6Aa}
Free / 12	6.7×10^{6Aa}	5.4×10^{6Aa}	4.9×10^{6Aa}
Microencapsulated/ 0	7.7×10^{6Aa}	2.1×10^{6Aa}	1.7×10^{6Aa}
Microencapsulated/12	2.5×10^{6Aa}	3.3×10^{6Aa}	3.9×10^{6Aa}

[A] Means with the same letter in the same line are not significantly different ($P<0.05$)
[a] Means with the same letter in the same column are not significantly different ($P<0.05$)

Stability of free and microencapsulated B. lactis in yoghurt

The viability of probiotic bacteria in yoghurt depends on the species used, the inter-reactions between the species present, the culture conditions, the chemical composition of the fermentation medium, the final acidity, the amount of milk solids, the availability of the nutrients, the presence of growth stimulants and/or inhibitors, the concentration of sugars, the quantity of dissolved oxygen (especially for *Bifidobacterium* spp.), the size of the inoculum, the incubation temperature, the fermentation time and the storage temperature (25, 26, 27, 28, 29).

The results of the survival of free and encapsulated *B. lactis* in yoghurt, can be found in **Tables 3** and **4**. The yoghurt containing free *B. lactis* showed a slight fall in pH, mainly in the first 7 d of storage, which probably resulted as a function of

residual fermentation. No significant alterations in pH occurred in the yoghurt containing the microencapsulated microorganism. The cultures used in this study probably did not cause over acidification under the conditions tested, which is an important factor in maintaining the quality of the product during its shelf life (30). The free and encapsulated *B. lactis* populations in the yoghurt, gradually declined by 7 logarithmic cycles up to 28 d of storage. The *L. bulgaricus* populations suffered a reduction of 2 logarithmic cycles, in both treatments, whilst the populations of *S. thermophilus* suffered a decline of 1 cycle after 14 and 21 d of storage (**Tables 3** and **4**). This result differed from that reported by Rybka & Kailasapathy (31), where the populations of *S. thermophilus, L. bulgaricus* and *Bifidobacterium* spp. suffered reductions of 1 cycle, 5 cycles and 2 cycles respectively, after 29 d of storage at 4°C in yoghurt with pH values between 4.49 and 4.10. However they are in agreement with those presented by Martin & Shou (30) where some species of *Bifidobacterium* suffered reductions of up to 5 logarithmic cycles in yoghurt at pH 4.2.

The number of viable bifidobacteria cells decreases in the presence of yoghurt cultures, possibly due to the presence of inhibitory substances, mainly hydrogen peroxide, produced by *L. delbrueckii* spp. *bulgaricus* and *S. salivarius* ssp. *thermophilus* (32, 33, 34).

Microencapsulation in cellulose acetate phthalate was not effective in protecting *B. lactis* in yoghurt, since the number of viable cells of the microencapsulated microorganism declined at the same rate as that of the free cells. The alterations in structure followed during the assays on acid pH resistance (**Figs 2a**, **2b** and **2c**), indicated that the microcapsules suffered significant morphological alterations

under these conditions, although even so they were efficient. Probably the extended permanence in the acid pH of the yoghurt, different from the 2 h permanence at the stomach pH, resulted in damage to the microcapsule walls, allowing for diffusion of H^+ or other inhibitory substances into the capsules, resulting in the death of the microorganisms. The encapsulation of *B. longum* in milk fat also failed to improve the survival of this culture in high acid yoghurt (19).

Table 3 Count of *B. lactis* (free), *S. thermophilus* and *L. bulgaricus* (cfu/ml) in yoghurt pH 4.2, during 28 d storage at 7°C

Storage (d)	pH	*B. lactis*	*S. thermophilus*	*L. bulgaricus*
0	4.20	1.1×10^8	1.5×10^8	2.0×10^8
7	3.98	3.5×10^6	1.1×10^8	1.5×10^8
14	3.92	4.2×10^4	1.4×10^8	4.0×10^7
21	3.94	6.9×10^3	4.5×10^7	1.0×10^7
28	3.92	1.3×10^1	1.0×10^7	7.1×10^6

Table 4 Count of *B. lactis* (microencapsulated), *S. thermophilus* and *L. bulgaricus* (cfu/ml) in yoghurt pH 4.2, during 28 d storage at 7°C

Storage (d)	pH	*B. lactis*	*S. thermophilus*	*L. bulgaricus*
0	4.23	1.0×10^8	8.5×10^8	5.5×10^8
7	4.28	4.6×10^6	5.5×10^8	1.3×10^8
14	4.29	8.2×10^4	1.3×10^8	1.2×10^7
21	4.27	8.0×10^3	5.5×10^7	3.5×10^7
28	4.28	1.0×10^1	3.9×10^7	1.0×10^6

Conclusions

Spray dried *B. lactis* and *L. acidophilus*, microencapsulated in cellulose acetate phthalate at levels recommended for ingestion, presented good potential for use as dietary aids, since they are resistant to acid and bile solutions and were liberated in conditions similar to those of the intestine. However, the use of high acid yoghurt

as a vehicle for the consumption of free or microencapsulated *B. lactis* was shown to be inadequate, due to the low survival rates obtained.

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CONCLUSÕES GERAIS

- *Lactobacillus acidophilus* (La-05) e *Bifidobacterium. lactis* (Bb-12) mostraram-se extremamente tolerantes à presença de bile em todas as condições testadas, o que é fundamental para o emprego desses como probióticos.
- A sobrevivência, e consequentemente as propriedades terapêuticas, de *L. acidophilus* e *B. lactis* ficariam comprometidas se o pH estomacal de quem os ingerissem estivesse abaixo de 2, uma vez que esses microrganismos são menos resistentes nessas condições, principalmente quando inoculados na forma livre ou imobilizados em alginato.
- Foi possível imobilizar *L. acidophilus* e *B. lactis* em alginato de cálcio, utilizando a técnica de coextrusão. O alginato não foi barreira efetiva contra às condições adversas presentes nas soluções de pH 1 e no iogurte, embora não tenha influenciado negativamente em nenhuma das condições testadas.
- *L. acidophilus* e *B. lactis*, livres e imobilizados, sobreviveram em bom número em leite acidificado. Porém, a população de *B. lactis* foi dizimada em iogurte, o que indica que nesse produto outros fatores podem ter influenciado na sobrevivência, não só o pH ácido.
- Foi possível microencapsular *L. acidophilus* e *B. lactis* em aceto fitalato de celulose, utilizando a técnica de "spray drying". Quanto menor a temperatura empregada no processo, maior a taxa de sobrevivência para ambos. Entretanto, *B. lactis* se adequou melhor ao processo porque foi mais resistente que *L. acidophilus* em todas as condições testadas, inclusive durante o armazenamento.
- A microcápsula de aceto fitalato de celulose foi eficiente quando os microrganismos foram inoculados no pH 1 e ineficiente quando esses foram incorporados no iogurte de alta acidez.