UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

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PERFIL TECNOLÓGICO E FUNCIONAL DE CEPAS PROBIÓTICAS EM LEITE FERMENTADO

São José do Rio Preto
2013

Sabrina Neves Casarotti

Perfil tecnológico e funcional de cepas probióticas em leite fermentado

Tese apresentada como parte dos requisitos para obtenção do título de Doutor junto ao Programa de Pós-Graduação em Engenharia e Ciência de Alimentos, área de Ciência e Tecnologia de Alimentos, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientador:

Profª. Drª. Ana Lúcia Barretto Penna

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2013

Casarotti, Sabrina Neves.
Perfil tecnológico e funcional de cepas probióticas em leite fermentado / Sabrina Neves Casarotti. -- São José do Rio Preto, 2013
174 f. : il., gráfs., tabs.

Orientador: Ana Lúcia Barretto Penna
Tese (doutorado) – Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências, Letras e Ciências Exatas

1. Tecnologia de alimentos. 2. Leite fermentado - Indústria. 3. Probióticos. I. Penna, Ana Lúcia Barretto. II. Universidade Estadual Paulista "Júlio de Mesquita Filho". Instituto de Biociências, Letras e Ciências Exatas. III. Título.

CDU – 637.146

Ficha catalográfica elaborada pela Biblioteca do IBILCE
UNESP - Campus de São José do Rio Preto

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São José do Rio Preto, 07 de outubro de 2013.

Dedico este trabalho à minha família: meus pais, Antônio e Solange, minha irmã Marília, meu esposo Bruno, por todo apoio, amor e carinho.

AGRADECIMENTOS

A Deus, pela força e direcionamento concedidos a mim para a preparação do projeto de Doutorado, execução e finalização deste trabalho.

À Prof. Dra. Ana Lúcia Barretto Penna pela confiança, oportunidades e ensinamentos e pela orientação deste trabalho.

Aos meus amigos do Laboratório de Leite e Derivados, alguns ainda presentes e outros que já fazem falta, Ana Beatriz Ceneviva, Aline Teodoro, Daniel Amaral, Íris Diamantino, Luana Faria, Raquel Gomes, Tatiane Ferreira e Vívian Diamantino, pela ajuda, conversas diárias e bons momentos compartilhados ao longo desses anos.

Às estagiárias, Andréia Queiroz e Taciana Marcon, pela inestimável ajuda na execução de etapas deste projeto.

Ao professor Roberto da Silva pelo uso do cromatógrafo e ao Diego Alves-Monteiro pela ajuda nas análises cromatográficas de ácidos orgânicos.

À Márcia Moretti pela ajuda na determinação de lactose.

Ao Bruno Carneiro pela ajuda no teste com as células Caco-2.

À Dra. Cristina Bogsan por ensinar como utilizar o CINAC.

À Dra. Flávia Carolina Alonso Buriti pelas sugestões dedicadas à realização dos ensaios de resistência dos probiótico *in vitro*.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela bolsa de doutorado concedida (processo n° 2009/12808-7).

Aos professores do Programa de Pós-Graduação em Engenharia e Ciência de Alimentos do IBILCE-UNESP pelos conhecimentos transmitidos.

Aos membros da banca pela contribuição para melhoria da qualidade deste trabalho.

À UNESP pela oportunidade de realização do curso de doutorado.

Ao meu esposo, Bruno, por seu amor e por estar sempre ao meu lado.

Aos meus queridos pais, Antônio e Solange, pelo carinho e incentivo em todos os momentos da minha caminhada, incondicionalmente. Vocês foram fundamentais para conclusão deste trabalho.

À minha irmã, Marília, pelo companheirismo por estar sempre presente em todos os momentos e novas etapas da minha vida.

A todos que, de alguma maneira, contribuíram para a realização deste trabalho.

RESUMO

Durante a estocagem de leites fermentados, as bactérias probióticas devem manter-se viáveis, para que resultem em benefício para a saúde do consumidor. A composição da cultura lática utilizada para a fermentação e os ingredientes adicionados ao leite influenciam a viabilidade dos probióticos durante a estocagem refrigerada. Além disso, é importante que os probióticos sobrevivam às condições adversas encontradas durante a passagem pelo trato gastrointestinal (TGI). A presença de medicamentos de diferentes tipos no TGI também pode suprimir ou reduzir o efeito terapêutico dos micro-organismos probióticos. O principal objetivo desse trabalho foi avaliar o efeito da composição da cultura e de diferentes matrizes na qualidade tecnológica de leite fermentado e o efeito de medicamentos comerciais sobre a sobrevivência dos probióticos. Para melhor distribuição e realização dos experimentos, o trabalho foi dividido em quatro partes.

Na primeira parte foi estudado o efeito da composição da cultura lática durante a fermentação, por meio da produção de ácidos orgânicos, consumo de lactose e cinética de acidificação, e durante a estocagem de leites fermentados, avaliando-se o pH, sinérese, viabilidade dos micro-organismos (*S. thermophilus, Lactobacillus acidophilus* e *Bifidobacterium animalis* subsp. *lactis*), sobrevivência dos probióticos às condições simuladas do TGI, características sensoriais dos produtos e concentração dos ácidos orgânicos. As bactérias que produziram a maior quantidade de ácido lático foram as homofermentativas *S. thermophilus* e *L. acidophilus*. Os produtos fermentados contendo *B. animalis* subsp. *lactis* apresentaram os maiores teores de ácido acético ao final da fermentação. Apenas *L. acidophilus* foi capaz de metabolizar o citrato, enquanto os teores de piruvato aumentaram ligeiramente durante a fermentação. A cinética de fermentação foi influenciada pela composição da cultura lática. Os maiores valores de pH ao final da estocagem foram obtidos para os tratamentos com as culturas probióticas. A sinérese reduziu durante a estocagem e o menor valor foi observado no tratamento com a cultura pura de *S. thermophilus*. As bifidobactérias apresentaram maior capacidade de sobrevivência durante o armazenamento e a viabilidade do lactobacilo foi maior na presença de *S. thermophilus*. A sobrevivência do lactobacilo ao

final do teste que simula as condições do TGI foi inferior à das bifidobactérias e houve redução da resistência de ambas as cepas probióticas ao ensaio *in vitro* durante o armazenamento refrigerado. Os produtos fermentados apenas com a cultura de *L. acidophilus* receberam as menores notas na avaliação sensorial, principalmente nos atributos sabor e aceitabilidade geral. Quanto maior a concentração de ácido lático, menores foram as notas recebidas para aceitabilidade geral e sabor, entretanto, as notas atribuídas para os parâmetros aceitabilidade geral e sabor foram maiores quanto maior a concentração de ácido cítrico nas amostras.

Na segunda parte, para estudar o efeito das diferentes matrizes, foi feita a adição das farinhas de banana, maçã e uva ao leite e a fermentação com a cultura ABT-4, composta por *S. thermophilus, L. acidophilus* e *B. animalis* subsp. *lactis*. O leite fermentado foi avaliado durante a acidificação e a estocagem refrigerada. A adição das farinhas não afetou o tempo final de fermentação e a acidez titulável ao final da estocagem, e os menores valores de pH foram obtidos nos tratamentos com adição de farinhas aos 28 dias de estocagem. A presença da farinha de banana aumentou as populações dos probióticos ao final da estocagem dos produtos, mas esse aumento não foi significativo do ponto de vista microbiológico, pois foram inferiores a 1 log UFC/mL. Além disso, as farinhas de frutas protegeram o *L. acidophilus* durante a simulação das condições do TGI.

Ainda para estudar o efeito das diferentes matrizes, numa terceira parte, a influência da farinha de quinoa foi avaliada durante a fermentação dos produtos e estocagem refrigerada por 28 dias. A farinha de quinoa nas concentrações de 2 e 3% prejudicaram a capacidade acidificante dos micro-organismos da cultura utilizada (ABT-4, composta por *S. thermophilus, L. acidophilus* e *B. animalis* subsp. *lactis*), mas não foi suficiente para aumentar o tempo final de fermentação. Os produtos adicionados de farinha de quinoa apresentaram os maiores valores de acidez titulável e menores valores de pH ao final da estocagem. Além disso, a adição de farinha de quinoa não afetou as populações dos probióticos do ponto de vista microbiológico, porque as diferenças foram inferiores a 1 log UFC/mL. No entanto, foi observada tendência no aumento da população da bifidobactéria no produto contendo 3% farinha de quinoa após 28 dias de armazenamento, por apresentar população 0,87 log UFC/mL superior ao controle. A farinha de quinoa não foi capaz de proteger os probióticos das condições

simuladas do TGI ao final do ensaio e não resultou em maior adesão dos probióticos às células Caco-2, uma vez que a diferença entre o tratamento controle e os suplementados foi inferior a 1 log UFC/mL. No entanto, a capacidade de adesão *in vitro* dos probióticos às células Caco-2 foi influenciada pela espécie do probiótico e pelo tempo de estocagem dos produtos.

Na quarta parte do trabalho, foram avaliados os efeitos *in vitro* de medicamentos de diversos grupos e do tipo de matriz (MRS, leite, leite adicionado de inulina) na sobrevivência das cepas probióticas La-5 e Bb-12. Alguns medicamentos usados no tratamento de doenças crônicas não-transmissíveis afetaram a sobrevivência dos probióticos, o que é relevante, visto que essas drogas podem acumular-se no intestino devido ao uso em longo prazo. Dentre os analgésicos, as cepas probióticas foram inibidas, principalmente, pela dipirona sódica. O metotrexato de sódio foi o que apresentou a menor concentração mínima inibitória dentre todos os medicamentos avaliados. Os probióticos apresentaram maior resistência às condições simuladas do TGI quando estavam incorporados em leite ou leite adicionado de inulina em comparação ao MRS.

Palavras-chave: produto lácteo fermentado, probiótico, viabilidade, resistência *in vitro* ao trato gastrointestinal, metabolismo.

ABSTRACT

During storage of fermented milk, probiotic bacteria must retain their viability in order to promote health benefits to the consumer. The composition of the culture used during fermentation and the ingredients added to milk can influence the viability of probiotics during refrigerated storage. Furthermore, it is important that probiotics survive to the harsh conditions during the passage through the gastrointestinal tract (GIT). The presence of different drugs in the GIT can also suppress or reduce the therapeutic effect of probiotic microorganisms. The main objective of this study was to evaluate the effect of the composition of the culture and different matrices on the quality of fermented milk and the effect of medications on the survival of commercial probiotics strains. The work was divided in four parts for better distribution and execution of experiments.

In the first part, it was studied the effect of the composition of culture during fermentation through the production of organic acids, lactose consumption and kinetics of acidification and during storage, evaluating the pH, syneresis, viability of microorganisms (*S. thermophilus , Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp.), survival of probiotics against simulated GIT conditions, sensory characteristics and concentration of the organic acids. The bacteria that produced the largest amount of lactic acid were the homofermentatives *S. thermophilus* and *L. acidophilus*. Fermented products containing *B. animalis* subsp. *lactis* showed the highest levels of acetic acid at the end of fermentation. Only *L. acidophilus* was able to metabolize citrate, while the levels of pyruvate rose slightly during fermentation. The kinetics of acidification was influenced by the composition of lactic acid culture. The higher pH values at the end of storage were obtained for treatments with probiotic cultures. The syneresis reduced during storage and the lowest value was observed in the treatment with the pure culture of *S. thermophilus*. Bifidobacteria showed higher survivability during storage and the viability of lactobacilli was higher in the presence of *S. thermophilus*. Lactobacilli was less resistant than the bifidobacteria to GIT simulated conditions and the resistance of the both probiotic strains to the *in vitro* assay decreased during storage. Fermented products with only *L. acidophilus* culture received

the lowest scores in sensory evaluation, especially for flavor and overall acceptability attributes. The higher the lactic acid concentration, the lower the score received for overall acceptability and flavor; however, sensory parameters were greater the higher the concentration of citric acid in the samples.

In the second part, the effect of different matrices was studied. Banana, apple and grape flours were added to milk and the fermentation was conducted using the ABT-4 culture, composed by *S. thermophilus, L. acidophilus* and *B. animalis* subsp. *lactis*. The fermented milk was evaluated during acidification and refrigerated storage. The addition of flour did not affect the fermentation time neither acidity at the end of storage. Lower pH values were obtained with addition of flour after 28 days of storage. The presence of the banana flour increased the population of probiotics at the end of the storage, but the increase was not significant from the microbiological point of view, since it was below 1 log CFU/mL. Moreover, the fruit flours protected *L. acidophilus* against GIT simulated conditions.

Still concerning the effect of different matrices, in the third part, the influence of the quinoa flour was evaluated during fermentation and refrigerated storage for 28 days. The quinoa flour at concentrations of 2 and 3% reduced the acidifying ability of microorganisms used (ABT-4 culture, composed of *S. thermophilus*, *L. acidophilus* and *B. animalis* subsp. *lactis*), but it was not enough to increase the final fermentation time. Products supplemented with quinoa flour showed higher titratable acidity and lower pH values at the end of storage. Furthermore, the addition of quinoa flour did not affect the population of probiotics from the microbiological point of view, because the differences between control and supplemented treatments were less than 1 log CFU/mL. Nevertheless, there was a trend towards the increase of the population of bifidobacteria in the product containing 3% quinoa flour at the end of storage, which presented population 0.87 log CFU/mL higher compared to control. The quinoa flour did not protect the probiotics against simulated GIT conditions and did not result in greater adhesion of probiotics to Caco-2 cells, since the difference between supplemented and control treatment was less than 1 log CFU/mL. However, *in vitro* adhesion of probiotics to Caco-2 cells was influenced by the probiotic strain and by the storage time of the products.

In the fourth part of the study, the *in vitro* effects of drugs belonging to different groups and the type of matrix (MRS, milk, milk added with inulin) on the survival of probiotic strains La-5 and BB-12 were evaluated. Some drugs used in the treatment of chronic diseases affected the survival of probiotic strains, which is relevant since these drugs may accumulate in the intestine due to long term use. Among analgesics, probiotic strains were inhibited mainly by dipyrone. Methotrexate sodium presented the lowest MICs of all the drugs evaluated. Probiotics showed higher resistance to simulated GIT conditions when they were incorporated into milk or milk added with inulin compared to MRS.

Keywords: fermented dairy product, probiotics, viability, *in vitro* resistance to gastrointestinal tract, metabolism.

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LISTA DE NOTAÇÃO E ABREVIATURAS

ANOVA	Análise de Variância
Bb	*Bifidobacterium animalis* subsp. *lactis*
Caco-2	*Epithelial colorectal adenocarcinoma cells*
CINAC	Cinetique d'Acidificacion
D1	1 dia após a fermentação com armazenamento a 4 °C
D14	14 dias após a fermentação com armazenamento a 4 °C
D21	21 dias após a fermentação com armazenamento a 4 °C
D28	28 dias após a fermentação com armazenamento a 4 °C
D7	7 dias após a fermentação com armazenamento a 4 °C
FMA	Leite fermentado adicionado de farinha de maçã
FMB	Leite fermentado adicionado de farinha de banana
FMC	Leite fermentado controle
FMG	Leite fermentado adicionado de farinha de uva
FMQ1	Leite fermentado adicionado de 1% de farinha de quinoa
FMQ2	Leite fermentado adicionado de 2% de farinha de quinoa
FMQ3	Leite fermentado adicionado de 3% de farinha de quinoa
G	Grama
G	Força g
G + C	Guanina mais citosina
h	Horas
H_2SO_4	Ácido sulfúrico
HCl	Ácido clorídrico
La	*Lactobacillus acidophilus*
LAB	Bactéria acidolática
M17 agar	Agar para a enumeração de *Streptococcus thermophilus*
µg	Micrograma
MIC	Concentração inibitória mínima
min	Minutos
mM	Milimolar

MRS	de Man, Rogosa and Sharpe
n	Número de repetições do experimento
PBS	Solução tampão de fosfato
pH_{Vmax}	pH no qual foi atingida a V_{max}
RSM	Leite em pó desnatado
RSMI	Leite em pó desnatado adicionado de inulina
St	*Streptococcus thermophilus*
StBb	co-cultura de *S. thermophilus* e *B. animalis* subsp. *lactis*
StLa	co-cultura de *S. thermophilus* e *L. acidophilus*
TGI	Trato gastrointestinal
$t_{pH4,6}$	Tempo no qual foi atingido o valor de pH de 4,6
$t_{pH5,0}$	Tempo no qual foi atingido o valor de pH de 5,0
t_{Vmax}	Tempo no qual foi atingida a V_{max}
UFC	Unidades formadoras de colônias
V_{max}	Velocidade máxima de acidificação
µL	Microlitro

SUMÁRIO

INTRODUÇÃO GERAL

Os leites fermentados são resultado da acidificação do leite por meio da atividade metabólica de bactérias acidoláticas (LAB), que promovem importantes modificações físico-químicas, sensoriais e microbiológicas nestes produtos. Para a fabricação de leites fermentados pode ser utilizada a cultura tradicional de iogurtes, constituída por *Streptococcus thermophilus* e *Lactobacillus delbrueckii* subsp. *bulgaricus,* que contribuem para as características sensoriais adequadas do produto. Entretanto, nos últimos anos, passou-se a adicionar bactérias probióticas nos leites fermentados. Estas bactérias, diferentemente daquelas da cultura tradicional, promovem efeitos benéficos à saúde do consumidor, se ingeridas regularmente na dieta, em quantidades adequadas.

Dentre as espécies de bactérias acidoláticas mais utilizadas como probióticas estão *Lactobacillus acidophilus* e *Bifidobacterium animalis.* Estas bactérias apresentam baixa atividade proteolítica e, por isso, desenvolvem-se lentamente no leite, fazendo com que o tempo de fermentação seja muito longo. Por esse motivo, usualmente são combinadas com os micro-organismos da cultura do iogurte, que facilitam seu desenvolvimento no leite por serem mais proteolíticos. No entanto, estudos demonstram que o *L. bulgaricus* prejudica a sobrevivência das bactérias probióticas em leites fermentados por ser uma espécie acidificante. Dessa forma, muitos produtos probióticos comercializados não contêm esta bactéria em sua formulação. Nesse caso, é empregado somente o *S. thermophilus* juntamente com as bactérias probióticas.

As espécies de bactérias acidoláticas podem conferir distintas e variadas características sensoriais aos leites fermentados. O efeito das bactérias acidoláticas sobre o aroma e sabor típicos de leites fermentados ocorre devido à formação de ácidos orgânicos, tais como os ácidos lático, acético e cítrico durante a fermentação e armazenamento dos produtos. O perfil de produção de ácidos orgânicos varia conforme a composição da cultura iniciadora utilizada, e portanto, esta variável pode influenciar na qualidade sensorial do produto.

Existem poucas informações a cerca das vias metabólicas seguidas pelos micro-organismos probióticos, como culturas puras ou em associação, durante a fermentação e estocagem dos leites fermentados. A manutenção da estabilidade dos probióticos e a obtenção de características sensoriais aceitáveis em leites fermentados podem ser aprimoradas conhecendo-se quais metabólitos são produzidos e em quais concentrações.

Assim, é possível selecionar aquelas combinações de culturas láticas mais vantajosas às características tecnológicas do leite fermentado e ao mesmo tempo, que permitam elevada sobrevivência dos probióticos, resultando em efeitos benéficos à saúde.

A fim de maximizar a eficácia dos micro-organismos probióticos, os leites fermentados podem ser adicionados de prebióticos. Dentre os ingredientes já reconhecidos como prebióticos, estão a inulina e a oligofrutose. No entanto, com o objetivo de atender à crescente demanda por produtos inovadores e saudáveis, torna-se interessante estudar novas fontes de ingredientes que possam estimular o crescimento e sobrevivência dos micro-organismos probióticos durante a fermentação e armazenamento de leites fermentados, assim como durante sua passagem pelo trato gastrointestinal.

Outro aspecto que deve ser levado em consideração é a interação entre os micro-organismos probióticos e os medicamentos. Sabe-se que muitas pessoas que consomem alimentos probióticos podem estar sendo, simultaneamente, submetidas a tratamentos medicamentosos e, até o presente momento, é desconhecido o efeito de medicamentos sobre a sobrevivência dos probióticos. Por isso, é importante avaliar se determinadas drogas podem influenciar na sobrevivência dos probióticos. Esses resultados irão fornecer informações adicionais sobre a viabilidade dos probióticos aos médicos, nutricionistas, farmacêuticos e pacientes quando se faz uso de medicamentos.

Com base nessas evidências, o principal objetivo desse trabalho foi avaliar o efeito da composição da cultura e de diferentes matrizes na qualidade tecnológica de leite fermentado e o efeito de medicamentos comerciais sobre a sobrevivência dos probióticos.

Os objetivos específicos foram:

a) avaliar o efeito de diferentes combinações de culturas láticas sobre:

- cinética de acidificação, produção de ácidos orgânicos (lático, acético, cítrico e fórmico) e sobre o consumo de lactose durante a fermentação (pH inicial; pH 6,0; pH 5,5; pH 5; pH 4,6) a 42 °C;
- sinérese, características sensoriais e produção de ácidos orgânicos, quinzenalmente, durante a estocagem do produto por 28 dias;
- sobrevivência das bactérias acidoláticas e bifidobactérias no produto, quinzenalmente, durante a estocagem por 28 dias;

- sobrevivência das bactérias probióticas frente às condições gastrointestinais por simulação *in vitro* em amostras de leite fermentado, quinzenalmente, durante a estocagem 28 dias.

b) avaliar o efeito da matriz contendo farinhas de banana, maçã e uva (1%), sobre:
- o perfil de acidificação durante a fermentação a 42 °C;
- pós-acidificação do produto, viabilidade das bactérias (*S. thermophilus, L. acidophilus* e *B. animalis* subsp. *lactis)*, e resistência dos probióticos às condições *in vitro* do trato gastrointestinal ao longo do armazenamento refrigerado, quinzenalmente, por 28 dias.

c) avaliar o efeito da matriz contendo farinha de quinoa, em diferentes concentrações (1-3%), sobre:
- o perfil de acidificação durante a fermentação a 42 °C;
- parâmetros físico-químicos, como pH e acidez, quinzenalmente, durante a estocagem por 28 dias;
- viabilidade das bactérias (*S. thermophilus, L. acidophilus* e *B. animalis* subsp. *lactis)*, quinzenalmente, durante o armazenamento por 28 dias;
- resistência dos probióticos às condições *in vitro* do trato gastrointestinal, quinzenalmente, e sua adesão às células intestinais da linhagem Caco-2, após 1 e 28 dias, em amostras de leite fermentado probiótico, durante o armazenamento 28 dias.

d) avaliar o efeito de medicamentos e da matriz na sobrevivência dos probióticos:
- efeito de medicamentos comerciais de diferentes grupos (analgésicos, anti-inflamatórios, anti-hipertensivos, etc) sobre o crescimento de probióticos e
- efeito do tipo de matriz contendo inulina sobre a resistência dos probióticos às condições *in vitro* do trato gastrointestinal.

O presente trabalho foi organizado em cinco capítulos para melhor distribuição e entendimento dos assuntos abordados. O Capítulo 1 consiste em uma Revisão de Literatura do tema abordado nessa tese. Os capítulos 2, 3, 4 e 5 foram redigidos na forma de artigos científicos para a publicação em revistas internacionais. O Capítulo 6 apresenta as conclusões gerais desse trabalho.

Capítulo 1

REVISÃO DE LITERATURA

1. Leite fermentado

Os produtos de leite fermentado ganharam importância devido ao seu excelente conteúdo nutricional e à possibilidade de serem adicionados de ingredientes funcionais (SÁNCHEZ et al., 2009). Por isso, esses produtos estão disponíveis em uma ampla variedade de texturas (por exemplo, líquido, firme, suave), de conteúdo gordura (integral, baixo teor de gordura, desnatado) e sabores (natural, frutas, cereais) (MCKINLEY, 2005). A imagem de alimento saudável é confirmada pela adição de várias preparações de frutas ao leite fermentado, incluindo os benefícios à saúde proporcionados pelas frutas, tais como o fornecimento de antioxidantes e fibras (O'RELL; CHANDAN, 2006). Nos últimos anos, leites fermentados produzidos utilizando-se extratos hidrossolúveis de soja (FERRAGUT et al., 2009; CRUZ et al., 2009c), de milho (SUPAVITITPATANA et al., 2008) e de amendoim (ISANGA; ZHANG, 2009) foram desenvolvidos como uma alternativa para vegetarianos ou aos consumidres que apresentam alergenicidade às proteínas do leite. Além disso, a inclusão de catequinas do chá para aumentar as propriedades antioxidantes e antimicrobianas (JAZIRI et al., 2009), também já foi considerada.

1.1. Definição, características e mercado

Os leites fermentados são produtos adicionados ou não de outras substâncias alimentícias, obtidos por coagulação e diminuição do pH do leite, ou leite reconstituído, acidificado por bactérias láticas, resultando em consistência e textura típicas, segundo os Padrões de Identidade e Qualidade (PIQ) de Leites Fermentados (BRASIL, 2000). A fermentação destes produtos se realiza com um ou vários dos seguintes cultivos: *Lactobacillus acidophilus, L. casei, Bifidobacterium* sp., *Streptococcus thermophilus* e/ou outras bactérias acidoláticas que, por sua atividade, contribuem para a determinação das características do produto final (BRASIL, 2000).

As principais características de qualidade dos leites fermentados incluem textura, sabor, aroma e odor (POURAHMAD; ASSADI, 2005). O leite fermentado é tipicamente caracterizado como um gel suave e viscoso com sabor ácido acentuado

característico. Este produto é rico em vitaminas e minerais e excelente fonte de cálcio e proteína. Além dos benefícios nutricionais, apresenta benefícios à saúde, como alívio da intolerância à lactose. Os efeitos benéficos dos leites fermentados podem ser potencializados pelo uso de micro-organismos probióticos em sua formulação (CHENG, 2010; ASHRAF; SHAH, 2011).

Estudos demonstram que a qualidade do leite fermentado pode ser afetada por diversos fatores, tais como o tipo de leite utilizado (KAMINARIDES; STAMOU; MASSOURAS, 2007; FLORENCE et al., 2009; FLORENCE et al., 2012), culturas *starter* (BESHKOVA et al., 1998; ABBASI et al., 2009), adoçantes (LUBBERS et al., 2007), prebióticos (ALTING et al., 2009; SINGH; KIM, 2009; RAMIREZ-SANTIAGO et al., 2010), e quantidade de sólidos totais (AMATAYAKUL; SHERKAT; SHAH, 2006; PENNA; CONVERTI; OLIVEIRA, 2006).

Com os progressos econômicos que o Brasil alcançou nos últimos anos, o consumo de alimentos, de um modo geral, se elevou (SIQUEIRA et al., 2010). De acordo com o Kantar WorldPanel, em 2011 o gasto médio do brasileiro com leite fermentado cresceu 14% em relação ao ano anterior, a penetração dessa categoria nos domicílios do país foi de 42%, e 67% do volume consumido no Brasil em 2011 concentrou-se nos lares com crianças.

1.2. Tecnologia de fabricação

A fabricação de leite fermentado usa uma técnica de preparo simples que se expande cada vez mais no mundo inteiro e que, atualmente, vem se transformando em um processo bastante sofisticado. Com a rápida incorporação desse produto aos hábitos alimentares, a competição industrial desencadeou a busca de novos processos que possibilitem a redução dos custos de fabricação sem prejuízo da qualidade do produto (GRANATO, 2007).

O leite fermentado pode ser obtido do leite de várias espécies de animais, sendo que as comumente utilizadas são de vaca, de cabra e de ovelha. O leite utilizado para fabricação de leite fermentado deve ser de boa precedência e qualidade, pois é responsável pelo seu valor nutricional e qualidade microbiológica. Deve apresentar ausência total ou presença mínima de substâncias estranhas, ausência de micro-organismos patogênicos, ser isento de antibióticos, acidez máxima de 20 a 24 ºD, sabor

e odor normais, extrato seco o mais elevado possível e ser proveniente de úberes sãos (SPREER; MIXA, 1998).

A padronização da gordura do leite é feita utilizando-se centrífugas separadoras, enquanto a padronização dos sólidos totais pode ser feita por concentração ou por adição de leite em pó desnatado ou outros ingredientes, até obter-se 15% de sólidos totais. Essa etapa melhora a consistência final do leite fermentado, diminuindo a sinérese no produto, reduzindo ligeiramente a produção de ácido durante a fermentação (VARNAM; SUTHERLAND, 1994). Em seguida, são adicionados os estabilizantes, açúcares e/ou edulcorantes e então, o leite é homogeneizado com os objetivos de melhorar o sabor, o corpo e a consistência do produto final (SPREER; MIXA, 1998). Essa etapa reduz o tamanho dos glóbulos de gordura e impede a formação de uma camada de lipídeos na superfície do leite durante a fermentação. Além disso, permite maior integração da gordura com as micelas de caseína durante a coagulação, resultando no aumento da viscosidade do leite e, conseqüentemente, estabilidade do leite fermentado durante a estocagem (WALSTRA; WOUTERS; GEURTS, 2006).

O leite para a fabricação do leite fermentado é sempre submetido a um tratamento térmico. Esse tratamento tem como objetivo destruir os micro-organismos patogênicos produtores de toxinfecções, como *Salmonella* e *Campylobacter*, e outros que podem competir com as bactérias láticas, dificultando seu crescimento (VARNAM; SUTHERLAND, 1994). Além disso, esta etapa resulta em um produto final com melhor textura, pois promove a desnaturação das proteínas do soro (principalmente a β-lactoglobulina e a α-lactalbumina), que reduzem a contração do coágulo da caseína, diminuindo, conseqüentemente, a sinérese (TAMIME; ROBINSON; LATRILLE, 2001). Esse processo também expulsa o oxigênio dissolvido no leite e reduz o potencial redox (Eh), criando, assim, um ambiente favorável para as bactérias durante a fermentação (CHANDAN; O'RELL, 2006).

O tratamento térmico varia desde a pasteurização rápida até um processo de *Ultra High Temperature* (UHT) (VARNAM; SUTHERLAND, 1994). A pasteurização é feita a 85 ºC por 20-30 minutos ou 90 a 95 °C por 10 ou 5 minutos. A escolha dos parâmetros a serem utilizados no tratamento térmico de leites fermentados tem se mostrado como fator importante para a posterior viabilidade das bactérias probióticas. Estudos mostraram que diferentes parâmetros usados para o tratamento térmico de iogurte (85 °C/30 min, 95 °C/5 min, e 95 °C/15min) suplementado com *L. acidophilus* e *Bifidobacterium* spp, alteraram a viabilidade das cepas probióticas, e a máxima

viabilidade para ambas as cepas probióticas foi alcancada quando se utilizou o binômio 95 °C/15 min. Os autores sugerem que esse fenômeno pode estar relacionado a maior redução do oxigênio presente (menor potencial redox) e liberação de aminoácidos provenientes do processo de desnaturação das proteínas do soro (MORTAZAVIAN et al., 2006a; MORTAZAVIAN et al., 2006b). Como as bactérias probióticas, em especial linhagens de bifidobacteria, apresentam reduzida capacidade proteolítica, complexa exigencia nutricional e metabolismo anaeróbio (SHAH, 1997), é natural que sejam beneficiadas com as mudanças originadas dos elevados parâmetros utilizados no tratamento térmico do leite.

Após o tratamento térmico, o leite para a produção do leite fermentado é resfriado até 42 ºC para a inoculação das culturas tradicionais e/ou probióticas (TAMIME; ROBINSON; LATRILLE, 2001). Para a fabricação de iogurtes é obrigatório que seja utilizada a cultura protossimbiótica composta por *S. thermophilus* e *L. delbrueckii* subsp. *bulgaricus*, que pode ser acompanhada ou não de outras espécies (BRASIL, 2000). No entanto, para leites fermentados, essa exigência não está prevista na legislação e podem ser usadas diversas composições de espécies de bactérias acidoláticas, incluindo as cepas probióticas.

A temperatura ótima de incubação para o crescimento das culturas é de 40-45 ºC. Embora bactérias probióticas apresentem temperatura ótima de crescimento na faixa de 37-40 °C (VASILJEVIC; SHAH, 2008), em leites fermentados incubados a 45 °C observou-se a manutenção da viabilidade quantitativamente superior ao recomendado para exercer benefícios à saude do consumidor (DONKOR et al., 2006; DONKOR et al., 2007).

O desenvolvimento de leites fermentados suplementados com bactérias probióticas requer obrigatoriamente total atenção na etapa de fermentação. As cepas microbianas escolhidas devem ser compatíveis entre si, evitando problemas de inibição pela produção de ácidos, peróxidos, bacteriocinas e outros produtos de seu metabolismo, que podem influenciar no rendimento do processo, no produto final, e na velocidade de acidificação. Problemas de inibição entre culturas probióticas e do iogurte têm sido descritos e não podem ser desprezados (VINDEROLA; MOCCHIUTTI; REINHEIMER, 2002).

A conversão de lactose do leite em ácido láctico pelas bactérias é a mudança mais significativa que ocorre durante a fermentação. Outros metabólitos, tais como o acetaldeído, acetona, ácido fórmico, diacetil, ácido acético e ácido propiónico são

também produzidos durante a fermentação e contribuem para o sabor característico do leite fermentado (CHANDAN; O'RELL, 2006). Durante o processo de fermentação, a caseína sofre modificações de acordo com a variação de pH: (a) pH 5,5-5,2: ocorrem desintegrações parciais, formando espaços vazios entre as micelas; (b) pH 5,2-4,8: ocorre contração do agregado de caseína, formando partículas maiores que as micelas originais; (c) pH $\leq$ 4,6: ponto isoelétrico da caseína, quando ocorre agregação das partículas de caseína, formando uma rede de proteínas que retém os constituintes do leite e o soro (TAMIME; ROBINSON, 1999).

Ao final da fermentação o coágulo deve apresentar pH entre 4,5 e 4,7 e uma concentração de ácido lático de 0,9%; o gel deve ser liso, brilhante, sem desprendimento de soro ou gases (TAMIME; ROBINSON, 1999). O controle do pH é importante no processo de fermentação, pois a separação do soro está diretamente relacionada com este parâmetro. Em produtos com pH maior que 4,6 a coalhada não é suficientemente formada, favorecendo a sinérese. Por outro lado, em produtos com pH menor do que 4,0, ocorre a separação do soro devido à redução da hidratação das proteínas e contração do coágulo (BRANDÃO, 1995).

Uma vez que o nível desejado de acidez tenha sido atingido, o leite fermentado é resfriado e pode receber as polpas e/ou frutas, sendo, em seguida, envasado. Para produtos probióticos, torna-se fundamental que a polpa ou suco da fruta que será utilizada não tenha substâncias que causem inibição do metabolismo da bactéria probiótica. Dessa forma, testes preliminares com diferentes concentrações da polpa devem ser realizados para verificação da sua compatibilidade com a linhagem a ser utilizada. Buriti et al. (2007) relatam a inibição de crescimento de *Lactobacillus acidophilus* em mousse adicionado de suco concentrado de maracujá e atribuem a perda de viabilidade a certos componentes presentes no suco da fruta. Segundo os autores, os possíveis compostos envolvidos na inibição são o ácido ascórbico, carotenóides, compostos aromáticos (tióis, terpenos, álcoois) e ésteres de ácidos graxos, como butanoato de etila e hexanoato de etila. Por outro lado, a adição de açaí, manga, morango, pêssego e banana, mostraram influencia positiva sobre a manutenção da população de micro-organismos probióticos durante o armazenamento (DONKOR; TSANGALIS; SHAH, 2007; BAKIRCI; KAVAZ, 2008; KAILASAPATHY; HARMSTORF; PHILLIPS, 2008; ALMEIDA et al., 2009; ESPÍRITO SANTO et al., 2010). O efeito inibitório ou estimulatório promovidos pela presença das frutas ou sucos parece estar relacionado às cepas probióticas utilizadas.

Após a incorporação das polpas e/ou frutas, o leite fermentado é armazenado a 4 °C, sendo que a faixa de temperatura pode variar de 0-10 °C. Como a elaboração do leite fermentado é um processo biológico, é necessário o uso da refrigeração (<10 °C) para controlar a atividade metabólica dos micro-organismos e suas enzimas. Além de reduzir a atividade metabólica da cultura, o resfriamento tem as funções de controlar a acidez do produto final e de prevenir a pós-acidificação (TAMIME; ROBINSON; LATRILLE, 2001).

Durante as primeiras 24 horas de armazenamento, diversas alterações ocorrem no leite fermentado. O nível de sinérese diminui como resultado da hidratação de micelas de caseína (LUCEY, 2004; SHAH, 2006). Além disso, a força do gel também aumenta à medida que a temperatura do leite fermentado diminui (GUYOMARC'H et al., 2003). Portanto, é muito importante armazenar o produto por 12 horas a 4 °C e evitar a manipulação mecânica para evitar sinérese excessiva. O aparecimento do sabor característico do leite fermentado ocorre durante as 12 horas posteriores ao resfriamento, proporcionando as características finais adequadas (TAMIME; DEETH, 1980). Geralmente, o leite fermentado tem uma vida útil de 4 a 7 semanas (CHANDAN; O'RELL, 2006).

A qualidade do leite fermentado pode ser avaliada por meio de sua composição química, propriedades microbiológicas e físicas. Em geral, os parâmetros químicos e microbiológicos são estabelecidos pela legislação de cada país. Por exemplo, a legislação brasileira (BRASIL, 2000) exige que as contagens de bactérias láticas viáveis no leite fermentado sejam de, no mínimo, 10^7 UFC/mL ou g do produto, e no caso de bifidobactérias, a população mínima deve ser de 10^6 UFC/mL ou g do um produto ao longo do período de armazenagem. O teor de proteína deve de no mínimo 2,9 g/100g, enquanto a acidez pode variar entre 0,6 a 2,0 g de ácido lático/100 g do produto durante a vida de prateleira. Não existe qualquer requisito legal para as propriedades físicas do produto. Em geral, o leite fermentado deve ser firme, com textura suave, livre de grânulos e sem sinérese sobre a superfície do produto (TAMIME; ROBINSON, 1999). Vários métodos, incluindo avaliações sensoriais e instrumentais, podem ser utilizados para avaliar as propriedades físicas de leites fermentados.

2. Bactérias acidoláticas e bifidobactérias

2.1. Taxonomia

As bactérias Gram-positivas são divididas em dois grandes grupos filogenéticos *Clostridium* e *Actinomycetes*, com base em comparações do conteúdo de guanina + citosina (G + C) da seqüência do DNA ribossomal. O grupo *Clostridium* abrange as bactérias cuja composição do DNA contém conteúdo do par G + C inferior a 50%, tais como as LAB. O grupo *Actinomycetes* compreende as bactérias com conteúdo G + C superior a 50%. As bactérias do gênero *Bifidobacterium* exibem um conteúdo relativamente elevado de guanina + citosina (G+C) e, portanto, pertencem ao grupo *Actinomycetes.* Apesar disto, as bifidobactérias compartilham características fisiológicas, bioquímicas e também alguns nichos ecológicos, como o TGI, com as LAB típicas (VASILJEVIC; SHAH, 2008).

As bactérias acidoláticas (LAB) são descritas como micro-organismos Gram-positivos, bacilares ou cocos, desprovidos de citocromo, não esporulantes e catalase-negativos. Compõem um grupo bastante heterogêneo quando se refere à capacidade de assimilar oxigênio, podendo ser anaeróbias, anaeróbias facultativas, aeróbias ou microaerófilas. De acordo com a temperatura ótima de desenvolvimento, são definidos como mesofílicos (20-40 ºC) ou termofílicos (40-50 ºC). São consideradas bactérias extremamente exigentes, pois necessitam de nutrientes específicos, como aminoácidos e vitaminas (SALMINEN; VON WRIGHT; OUWEHAND, 2004). Caracterizam-se por apresentar alta tolerância a ácidos**.** São micro-organismos estritamente fermentadores, produzindo ácido lático como o principal produto metabólico (HOLZAPFEL et al., 2001). Atualmente, são considerados do grupo das LAB os micro-organismos dos gêneros *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Mlissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weisella* (CROWLEY; MAHONY; VAN SINDEREN, 2013).

2.1.1. *Streptococcus thermophilus*

Streptococcus thermophilus é considerada a segunda espécie mais importante do grupo das bactérias acidoláticas com aplicação industrial, após *Lactococcus lactis*, com

um valor de mercado de cerca de 40 bilhões de dólares nos EUA (IYER et al., 2010). Apesar de *S. thermophilus* fazer parte do gênero *Streptococcus* (atualmente conhecido por ter mais de 40 espécies), que inclui vários patógenos, é reconhecido como seguro para o consumo humano (*Generally Recognized as Safe* - GRAS) nos EUA e na Europa, devido a uma longa história de uso seguro na produção de alimentos.

A comparação dos genomas de *S. thermophilus* com genomas já publicados de estreptococos patogênicos destaca o seu parentesco com espécies patogênicas, mas também revela que os determinantes mais importantes para a patogenicidade estão ausentes ou presentes como pseudogenes, a menos que eles codifiquem funções celulares básicas. Isso reforçou a visão de que o consumo desta espécie por seres humanos não representa risco à saúde. A análise genômica comparativa mostra que a evolução moldou o genoma de *S. thermophilus*, principalmente, por meio de eventos de perda de função, revelando que os estreptococos usados em produtos lácteos seguiram um caminho evolutivo divergente ao das espécies patogênicas. Por outro lado, os estreptococos patogênicos são reconhecidos por uma elevada capacidade de liberar proteínas em sua superfície, a fim de conseguir a adesão celular ou escapar ao sistema imune do hospedeiro. O *S. thermophilus* perdeu esta característica única, bem como muitas funções relacionadas com a virulência (HOLS et al., 2005). Vários dos genes encontrados em *S. thermophilus* provavelmente se originaram de outras espécies de bactérias láticas, como *L. lactis* e *L. delbrueckii*, e assim, contribuem para sua adaptação ao ambiente do leite (DE VUYST; TSAKALIDOU, 2008; DELORME, 2008).

S. thermophilus tem morfologia de cocos (Figura 1), geralmente dispostos em cadeia curta. Cresce em temperaturas entre 37 e 45 °C e toleram temperaturas acima de 50 °C. Apresenta alta sensibilidade ao cloreto de sódio e algumas cepas produzem exopolissacarídeos, compostos benéficos à consistência de iogurtes e leites fermentados, mantendo a textura e a viscosidade durante o armazenamento. A espécie é de grande importância para a indústria de alimentos uma vez que é amplamente utilizado para a fabricação de produtos lácteos (HOLS et al., 2005). Além do uso tradicional em combinação com *L. delbrueckii* subsp. *bulgaricus* em iogurte, *S. thermophilus* é usado para produzir diversas variedades de queijo, como queijo suíço, Parmesão, Provolone, Mussarela e Asiago (FOX et al., 2004). Um dos papéis principais de *S. thermophilus* na fermentação do leite é promover a acidificação rápida. Além de ácido lático, também produz baixos teores de formato, acetoína, acetaldeído, diacetil e acetato como produtos finais (OTT; GERMOND; CHAINTREAU, 2000).

Figura 1. Morfologia de bactérias pertencentes à espécie *Streptococcus thermophilus*. (Fonte: http://microbewiki.kenyon.edu/index.php/Streptococcus_thermophilus).

S. thermophilus tem uma capacidade limitada para utilizar carboidratos, e a função principal de *S. thermophilus* na fermentação industrial de leite é a conversão de lactose em lactato em temperaturas elevadas. *S. thermophilus*, ao contrário de muitas outras bactérias Gram-positivas, prefere lactose a glicose como sua principal fonte de carbono e energia. A maioria das cepas de *S. thermophilus* é incapaz de metabolizar a galactose e, portanto, libera esse açúcar para o meio durante a fermentação da lactose (MORA et al., 2002; DE VIN et al., 2005; VAILLANCOURT et al., 2008).

2.1.2. *Lactobacillus acidophilus*

Os lactobacilos possuem importante valor comercial para a indústria alimentícia, devido ao seu emprego na produção de lácteos fermentados, como culturas iniciadoras de fermentação na fabricação de queijos e em leites fermentados devido ao seu apelo funcional (VASQUEZ et al., 2005).

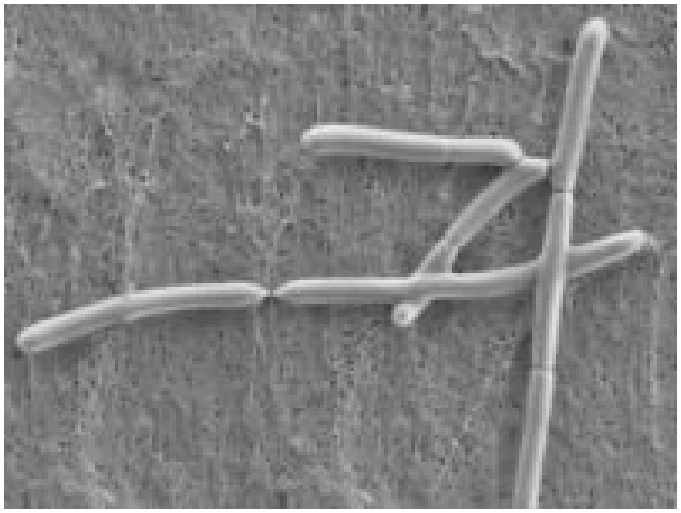

Figura 2. Morfologia de bactérias pertencentes à espécie *Lactobacillus acidophilus.* (Fonte: http://www.chr-hansen.com/products/product-areas/probiotics-for-dietary-supplements/strains/la-5r.html).

O *L. acidophilus* é um bacilo Gram-positivo com pontas arredondadas, incapaz de formar esporo, desprovido de flagelos, se encontra na forma de células livres, aos pares ou em cadeias curtas, com tamanho típico de 0,6-0,9 μm de largura e 1,5-6,0 μm de comprimento. Esta espécie apresenta algumas particularidades, tais como, ser pouco tolerante à salinidade, microaerofílica e com melhor desenvolvimento em meios sólidos pela anaerobiose ou pressão reduzida de oxigênio. As condições ótimas para a sua multiplicação eficaz são temperaturas de 35 a 40 ºC e valores de pH de 5,5 a 6,0. Porém, o desenvolvimento de *L. acidophilus* pode ocorrer a 45 ºC, e sua tolerância à acidez do meio varia entre 0,3 e 1,9 % (v/v) de ácido lático. Como micro-organismo homofermentativo, produz quase exclusivamente ácido lático a partir da degradação da glicose pela via de Embden-Meyerhof-Parnas (1,8 moles por mol de glicose), embora também possa produzir acetaldeído (GOMES; MALCATA, 1999).

Encontrado naturalmente na microbiota intestinal e no trato urogenital humano, *L. acidophilus* apresenta como principais funções: proteção contra patógenos, auxílio na hidrólise da lactose, aumento do valor nutricional dos alimentos devido à síntese de nutrientes (vitaminas B_6 e B_{12}, niacina, riboflavina e ácido fólico), estimulação da resposta imune intestinal e regulação dos níveis de colesterol no organismo (CAPOZZI et al., 2012; SINGH; AMDEKA; SINGH, 2012; AGGARWAL; SWAMI; KUMAR, 2013).

Dentre as cepas probióticas de *Lactobacillus acidophilus,* a La-5 é uma das mais amplamente utilizadas atualmente. Foi testada em vários ensaios clínicos em doses de até 50 bilhões de UFC/dia sem efeitos secundários relatados. Estudos já demonstraram que a cepa de *L. acidophilus* La-5 pode melhorar os níveis de colesterol total e da fração LDL (LDL-c) em indivíduos portadores de Diabetes do tipo 2 (EJTAHED et al., 2011). Outro estudo relatou que houve supressão de *Helicobacter pylori* após a ingestão de iogurte contendo *L. acidophilus* La-5 e *B. animalis* subsp. *lactis* BB-12, duas vezes ao dia, durante seis semanas (WANG et al., 2004).

2.1.3. *Bifidobacterium animalis* subsp. *lactis*

As bifidobactérias foram isoladas e identificadas pela primeira vez por Tissier (1900), a partir de fezes de recém-nascidos amamentados somente com leite materno (VASILJEVIC; SHAH, 2008). Esses micro-organismos são bastonetes Gram-positivos, não-esporulados, imóveis, catalase-negativos, não produtores de gás (com exceção de *B. indicum* e *B. asteroides*) e anaeróbios, habitantes característicos do intestino grosso e devido à sua morfologia "bifurcada" em forma de Y (Figura 3) foram denominados de *Bifidobacterium* (GOMES; MALCATA, 1999). Bifidobactérias fazem parte de um importante grupo de bactérias comensais do intestino humano, representando cerca de 3-7% da microbiota em adultos e, de acordo com alguns relatos, até 91% em recém-nascidos (MARTINEZ et al., 2013).

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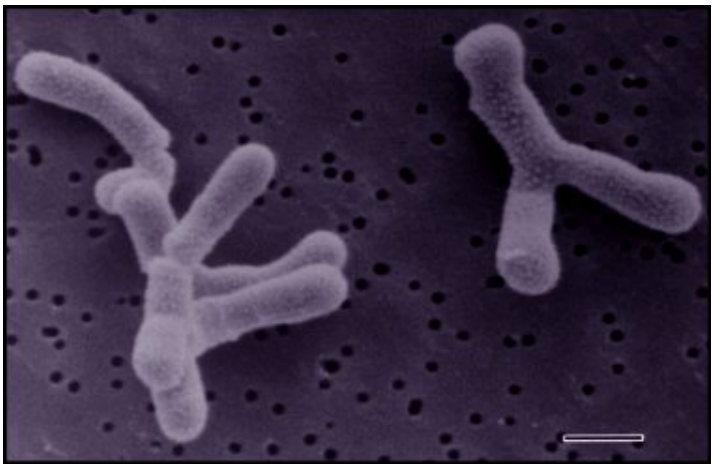


Figura 3. Morfologia de bactérias pertencentes à espécie *Bifidobacterium animalis.* (Fonte: http://microbewiki.kenyon.edu/index.php/Bifidobacterium).

A temperatura ótima desenvolvimento de bifidobactérias oscila entre 37 e 41 ºC, ocorrendo desenvolvimento máximo e mínimo entre 43 e 45 ºC e 25 e 28 ºC, respectivamente, e o pH ótimo entre valores 6,0 e 7,0, com ausência de desenvolvimento em valores de pH ácidos, de 4,5 a 5,0 ou em valores de pH alcalinos, de 8,0 a 8,5 (GOMES; MALCATA, 1999; CRONIN et al., 2011).

Devido à sua natureza fastidiosa, esses micro-organismos são difíceis de isolar e cultivar em laboratório. A taxonomia das bifidobactérias passa continuamente por mudanças, desde o seu primeiro isolado. Eles haviam sido designados inicialmente como pertencentes aos gêneros *Bacillus*, *Lactobacillus*, *Nocardia* e *Corynebacterium*. Atualmente, o gênero *Bifidobacterium* contém trinta e duas espécies, sendo as de origem humana: *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. dentium, B. infantis, B. longum* e *B. pseudocatenulatum.* De maneira geral, as bifidobactérias são capazes de produzir ácido lático, acético e pequena quantidade de ácido fórmico, diminuindo o pH do cólon e inibindo a proliferação de patógenos (VASILJEVIC; SHAH, 2008; RUSSELL et al., 2011).

A cepa *B. animalis* subsp. *lactis* BB-12 configura entre as cepas mais utilizadas como probióticos em alimentos. É reconhecida por possuir potente eficácia sobre a saúde humana, em se tratando de situações relacionadas à diarréia causada por rotavírus, má absorção da lactose, diarréia associada ao uso de antibióticos e diarréia associada ao *Clostridium difficile* (SHAH, 2007; GUEIMONDE et al., 2010). A sobrevivência das bifidobactérias em iogurtes e leites fermentados varia de acordo com a espécie, mas em geral, esses micro-organismos possuem características tecnológicas de interesse para a indústria alimentícia, que a tornam aptas para serem empregadas em diversos alimentos, tais como a tolerância à presença de oxigênio e aos ácidos (AKALIN; FENDERYA; AKBULUT, 2004).

2.2. Sistema metabólico

A característica essencial do metabolismo das LAB e das bifidobactérias é a fermentação de carboidratos juntamente com a fosforilação do substrato. A molécula de adenosina trifosfato (ATP) gerada é posteriormente utilizada para propósitos biossintéticos. As LAB apresentam uma enorme capacidade de degradar diferentes carboidratos e compostos relacionados. O produto final é geralmente o ácido lático,

correspondendo a mais de 50% do total produzido (SALMINEN; VON WRIGHT; OUWEHAND, 2004; RAVYTS; DE VUYST; LEROY, 2012).

Os membros do grupo das LAB são subdivididos em dois grupos distintos de acordo com o metabolismo do carboidrato que apresentam. O grupo das bactérias homofermentativas abrange os gêneros *Lactococcus, Pediococcus, Enterococcus, Streptococcus* e alguns lactobacilos, que utilizam a via Embden-Meyerhof-Parnas (glicolítica) para transformar o carbono principalmente em ácido lático. Por outro lado, as bactérias heretofermentativas utilizam a via da fosfoacetolase para produzir além do ácido lático, CO_2, etanol, acetato e citrato a partir da glicose. Os membros desse grupo incluem *Leuconostoc, Weisella* e alguns lactobacilos (VASILJEVIC; SHAH, 2008).

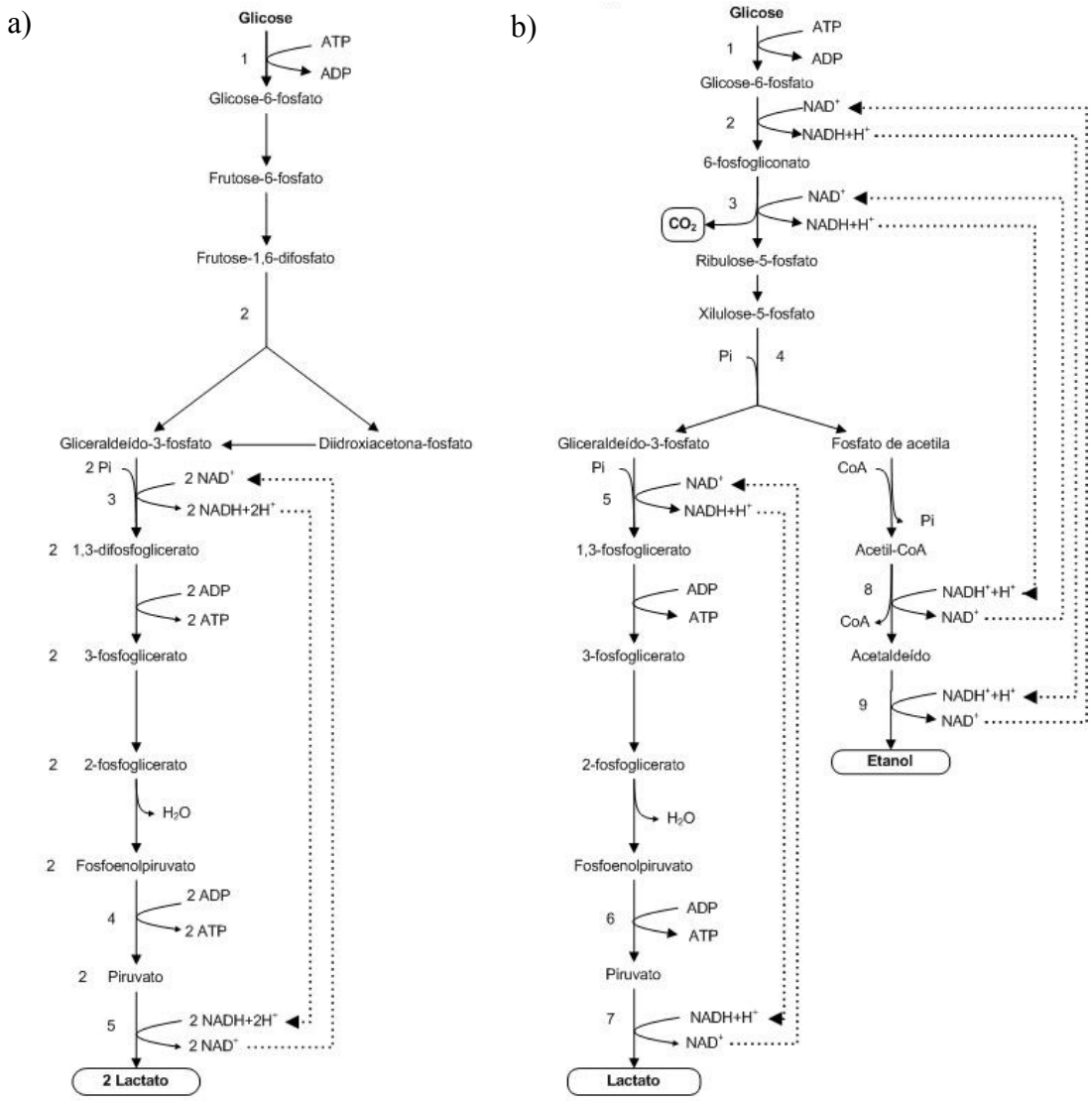
Várias condições de desenvolvimento podem modificar significativamente a formação do produto final de algumas bactérias de acidoláticas, o que pode alterar os produtos finais formados. Essas mudanças podem ser atribuídas a uma alteração do metabolismo do piruvato e/ou a utilização de receptores de elétrons externos, tais como oxigênio, em respirações anaeróbia ou aeróbia, ou compostos orgânicos (SALMINEN; VON WRIGHT; OUWEHAND, 2004).

As LAB, além das enzimas glicolíticas, também produzem enzimas proteolíticas e lipolíticas, que hidrolisam os nutrientes presentes no leite, que contribuem para a formação de compostos voláteis e modificação das características sensoriais do produto. Portanto, as LAB destacam-se pela grande importância industrial para a produção de derivados lácteos (LIU; HAN; ZHOU, 2011).

2.2.1. Metabolismo das hexoses

Os monossacarídeos podem ser metabolizados por duas vias: glicolítica ou fosfocetolase (Figuras 4a e 4b). A via glicolítica é caracterizada pela transformação da glicose em frutose-1,6-difosfato (FDP), produto intermediário chave da via, que é dividido por uma aldolase em dihidroxiacetona-fosfato (DHAP) e gliceraldeído-3-fosfato (GAP). Na via GAP, estes compostos são convertidos em piruvato em uma sequência metabólica que inclui fosforilação do substrato em dois sítios. Sobre condições normais, ou seja, excesso de açúcar e baixo teor de oxigênio, o piruvato é reduzido a ácido lático por uma lactato desidrogenase dependente da molécula de nicotinamida adenina dinucleotídeo (NAD^+), oxidando a forma reduzida do NAD (NADH) formado durante as etapas iniciais da via glicolítica. Dessa forma, obtém-se o

balanço redox, o ácido lático é praticamente o único produto final e o metabolismo é chamado de fermentação homolática. Essa fermentação resulta em ganho líquido de duas moléculas de ATP e duas moléculas de ácido lático por molécula de glicose fermentada (SALMINEN; VON WRIGHT; OUWEHAND, 2004).



Adaptado de Salminen, Von Wright e Ouwehand (2004).

Figura 4. Vias de fermentação das hexoses. a – Fermentação homolática (via glicolítica de Emden-Meyerhof-Parnas). 1: glicoquinase; 2: frutose 1,6-difosfato aldolase; 3: gliceraldeído-3-fosfato desidrogenase; 4: piruvato quinase; 5: lactato desidrogenase. b – Fermentação heterolática (via da 6-fosfogluconato/fosfocetolase). 1: glicoquinase; 2: glicose-6-fosfato desidrogenase; 3: 6-fosfogliconato desidrogenase; 4: fosfocetolase; 5: gliceraldeído-3-fosfato desidrogenase; 6: piruvato quinase; 7: lactato desidrogenase; 8: acetaldeído desidrogenase; 9: álcool desidrogenase.

A outra via de fermentação, a 6-fosfogluconato/fosfocetolase (6-PG/PK), é caracterizada pela desidrogenação inicial, com a formação de 6-fosfogliconato, seguido de descarboxilação. A pentose-5-fosfato resultante é dividida pela fosfocetolase em GAP e fosfato de acetila. O GAP é metabolizado da mesma forma que ocorre na via glicolítica, resultando na formação de ácido lático. Quando não existe receptor adicional de elétron disponível, o fosfato de acetila é reduzido a etanol via acetil-CoA e acetaldeído (SALMINEN; VON WRIGHT; OUWEHAND, 2004).

Essa fermentação é denominada heterolática, porque permite a formação de quantidades significantes de outros produtos finais da fermentação, como CO_2 e etanol, além do ácido lático, e resulta em ganho líquido de uma molécula de ATP e uma molécula de cada um dos produtos finais (ácido lático, etanol e CO_2) por molécula de glicose fermentada (SALMINEN; VON WRIGHT; OUWEHAND, 2004).

As bifidobactérias são organismos heterofermentativos com uma via metabólica especial, denominada via bifidus ou via frutose-6-fosfato, que permite a produção de ácidos acético e lático na proporção molar de 3:2. A enzima chave desta via metabólica fermentativa é a frutose-6-fosfato fosfocetolase (F6PPK), e por isso, pode ser usada como marcador taxonômico na identificação do gênero, mas não permite a diferenciação entre as espécies. A presença dessa enzima e a ausência das enzimas aldolase e glicose-6-fosfato desidrogenase diferenciam o gênero *Bifidobacterium* do gênero *Lactobacillus* (SALMINEN; VON WRIGHT; OUWEHAND, 2004; OLIVEIRA et al., 2012).

2.3. Influência da cultura lática sobre as características sensoriais de leites fermentados

O perfil sensorial do leite fermentado é influenciado diretamente pela atividade metabólica da bactéria utilizada, que interage intensamente com o meio ao converter determinados componentes em produtos metabólicos durante seu crescimento, tais como os ácidos não-voláteis, ácidos voláteis, compostos carbonílicos e outros compostos, como os aminoácidos (HELLER, 2001; SERRA et al., 2009).

Os compostos do aroma e sabor são formados pela hidrólise das proteínas do leite, gordura e lactose. A lactose é metabolizada em compostos aromáticos, como os aldeídos, alcoóis e ácidos carboxílicos por enzimas fosfotransferases das bactérias acidoláticas. A caseína é metabolizada por proteases e aminotransferases para produzir aminoácidos

livres e compostos aromáticos (GALLARDO-ESCAMILLA; KELLY; DELAHUNTY, 2005; OMAE; MAEYAMA; NISHMURA, 2008). A gordura sofre lipólise ou oxidação durante a fermentação. Quantidades consideráveis de ácidos graxos de cadeia curta são produzidos a partir de ácidos graxos saturados. Os ácidos graxos insaturados são oxidados na presença de radicais livres, originando os hidroperóxidos, que se decompõem rapidamente para formar hexanal ou aldeídos insaturados (CHENG, 2010).

Uma vez que as espécies de bactérias acidoláticas possuem diferentes sistemas metabólicos, os produtos lácteos fermentados por diferentes combinações de culturas iniciadoras apresentam características sensoriais distintas. Apesar de existirem diversos estudos sobre a produção de ácidos orgânicos em iogurtes, estão disponíveis poucos dados sobre a proporção e a concentração desses compostos em leites fermentados probióticos ao longo do armazenamento, sobretudo aqueles contendo *L. acidophilus* e *Bifidobacterium* sp.

2.3.1. Principais metabólitos que afetam as características sensoriais de leites fermentados

Os compostos que influenciam as características sensoriais do leite fermentado podem ser divididos em quatro categorias principais (TAMIME; ROBINSON, 1999):

- ácidos não-voláteis (lático, pirúvico, oxálico, succínico);
- ácidos voláteis (acético, propiônico, butírico);
- compostos carbonílicos (acetaldeído, acetona, acetoína, e diacetil) e
- outros compostos (certos aminoácidos e/ou constituintes formados por degradação térmica de proteína, gordura, e lactose).

Os ácidos orgânicos são os principais metabólitos presentes nos leites fermentados, encontrados em maior quantidade, e influenciam diretamente nas características sensoriais do produto. Os ácidos orgânicos mais encontrados nos leites fermentados durante a fermentação e a estocagem são orótico, cítrico, pirúvico, acético, propiônico, úrico e lático.

Østlie, Helland e Narvhus (2003) observaram diferentes perfis de produção de ácidos orgânicos por culturas puras de *B. animalis* BB-12*, L. acidophilus* 1748*, L. acidophilus* La-5, *L. rhamnosus* e *L. reuteri,* utilizando inóculo de 1% para preparação dos leites fermentados, ao longo de 72 horas de incubação a 37 °C. *L. acidophilus* 1748*, L. acidophilus* La-5 produziram a maior quantidade de ácido lático e *B. animalis* BB-12

a menor quantidade. Por outro lado, os maiores teores de ácido acético foram detectados nos leites fermentados inoculados com BB-12. Por produzirem ácido acético, as bifidobactérias podem conferir ao produto um sabor indesejável de vinagre. Por este motivo, são usadas em combinação com outras espécies de bactérias láticas, como o *S. thermophilus*, geralmente na proporção de 1:1. Houve aumento do conteúdo de piruvato durante as primeiras 20 horas de fermentação para todos os micro-organismos avaliados e, após esse período, a produção reduziu. A cepa que produziu maior quantidade foi *L. acidophilus* 1748. Foi observado ainda que, durante a fermentação, a concentração do ácido úrico manteve-se estável, ocorreu redução do ácido orótico e aumento do ácido succínico. Três cepas apresentaram a capacidade de metabolizar o citrato presente no leite inicial, mas a velocidade variou entre elas. *L. acidophilus* 1748*, L. acidophilus* La-5 metabolizaram praticamente todo o citrato após 20 e 48 horas de incubação, respectivamente.

La Torre, Tamime e Muir (2003) investigaram a produção de ácidos orgânicos em leites fermentados contendo bactérias probióticas e constataram a presença dos seguintes ácidos: orótico, cítrico, pirúvico, lático, úrico, acético, hipúrico. Durante o armazenamento, foram constatadas diferenças nas concentrações de ácido orótico entre os tipos de leites fermentados elaborados, sendo que os produtos contendo *S. thermophilus* apresentaram maiores quantidades deste ácido. Os leites fermentados por AB (*L. acidophilus, B. bifidum* e *B. lactis*) e AC/BL (*L. acidophilus, B. longum* e *B. infantis*), os únicos sem a adição de *S. thermophilus,* apresentaram valores bastante superiores na concentração de ácido acético em relação aos demais leites fermentados.

O crescimento e metabolismo de *B. animalis* (Bl) e *S. thermophilus* (St)*,* em culturas puras ou em co-cultura (St-Bl), em leite fermentado sem ou com a adição de 4% de inulina, foi avaliado por Oliveira et al. (2012). A presença de inulina aumentou os teores de ácidos láctico e acético e os compostos voláteis, demonstrando um efeito positivo simbiótico entre o pré e os probióticos. Em particular, a co-cultura St-Bl mostrou concentrações finais de ambos os micro-organismos cerca de 15 e 38% maior do que em suas respectivas culturas puras, evidenciando, assim, um claro efeito sinérgico entre estes micro-organismos. As concentrações de ácido láctico liberadas por St (11,0 g/L), Bl (8,1 g/L) e St-Bl (9,5 g/L) nas amostras contendo inulina foram, respectivamente, cerca de 15, 7 e 7% maior do que as obtidas na ausência de inulina, enquanto as de ácido acético foram cerca de 17% (St-Bl) e 23% (Bl) superiores nesses produtos. No que diz respeito à relação entre os ácidos acético/láctico, houve um pequeno aumento (2,70:2) devido à

adição de inulina. Concentrações crescentes desses compostos nos produtos finais elaborados com co-culturas poderiam ser atribuídas às alterações metabólicas induzidas pelo prebiótico, e podem ser explorados em nível industrial para aumentar a sua presença em produtos lácteos fermentados.

3. Produtos lácteos funcionais

Atualmente, devido aos avanços da ciência e ao fácil acesso à informação, os consumidores passaram a se preocupar mais com as questões de saúde e, por isso, buscam alimentos que contribuam para uma vida mais saudável. Os produtos lácteos representam um grande potencial de investimento para a indústria de alimentos, que pode ser explorado por meio do desenvolvimento de ingredientes, processos e produtos inovadores. Uma das principais tendências que impulsionou o crescimento do consumo de produtos lácteos nas últimas décadas foi o surgimento dos produtos funcionais.

Os alimentos funcionais são aqueles que, além de fornecerem a nutrição básica, promovem a saúde e/ou que possuem potencial para promover a saúde por meio de mecanismos não previstos pela nutrição convencional, sendo que esse efeito restringe-se à promoção da saúde e não à cura de doenças, devendo ser seguro para consumo sem a supervisão médica (GRANATO et al., 2010).

A legislação brasileira define alegação de propriedade funcional e alegação de propriedade de saúde, mas não define alimento funcional. Além disso, estabelece as diretrizes para sua utilização, bem como as condições de registro para os alimentos com alegação de propriedade funcional e/ou de saúde. Dentre as diretrizes para esse tipo de alimento, são permitidas alegações funcionais relacionadas com o papel fisiológico no crescimento, no desenvolvimento e nas funções normais do organismo e ainda alegações sobre a manutenção geral da saúde e a redução de risco de doenças. Não são permitidas alegações que façam referência à cura de doenças (BRASIL, 1999a; 1999b; 1999c).

A Agência Nacional de Vigilância Sanitária (ANVISA) aprovou uma Lista de Alimentos com Alegações de Propriedades Funcionais e/ou de Saúde, Novos Alimentos/Ingredientes, Substâncias Bioativas e Probióticos (BRASIL, 2008). Nessa lista, constam as alegações e os requisitos específicos dos seguintes ingredientes alimentícios: ácidos graxos (ômega 3), carotenoides (licopeno, luteína e zeaxantina), fibras alimentares (beta glucana, dextrina resistente, frutooligossacarídeo - FOS, goma

guar parcialmente hidrolisada, inulina, lactulose, polidextrose, psillium ou psyllium e quitosana), fitoesteróis, polióis (manitol, xilitol e sorbitol), probióticos e proteínas de soja (concentrado proteico de soja, isolado proteico de soja, proteína texturizada de soja).

Para se obter o registro de um alimento com alegação de propriedades funcionais e/ou de saúde, deve ser formulado um relatório técnico científico bastante detalhado, comprovando os benefícios e a segurança de uso do alimento. O conteúdo da propaganda desses produtos não pode ser diferente em seu significado, daquele aprovado para a rotulagem. As alegações devem ainda, estar em consonância com as diretrizes da política pública de saúde (BRASIL, 1999a; 1999b; 2004).

O crescente interesse dos consumidores pela relação entre nutrição, saúde e bem-estar é o principal motivo do sucesso do mercado de alimentos funcionais. Na América do Norte, até 93% dos consumidores acreditam que determinados alimentos têm benefícios para a saúde e podem reduzir o risco de doenças (CHAMPAGNE; MOLLGAARD, 2008). Embora a América Latina seja considerada um mercado emergente para os alimentos funcionais e produtos naturais, os fatores culturais, os baixos níveis de conhecimentos sobre nutrição e a renda limitam a entrada de tais produtos. Entretanto, nos últimos anos houve aumento na demanda por esse tipo de alimento (GRANATO et al., 2010).

Os maiores mercados para alimentos funcionais e suplementos são os Estados Unidos, Europa e Japão. No Brasil, este mercado cresce de maneira vertiginosa; são US$ 4 bilhões ao ano, segundo dados da consultoria Euromonitor. Em âmbito mundial, esse mercado faturou US$ 150 bilhões em 2011 e deverá ter um crescimento de 38% até 2017, com previsão de atingir US$ 207 bilhões(GRANATO et al., 2010b).

Os probióticos são os principais componentes bioativos de produtos lácteos fermentados funcionais e numerosos indicadores econômicos mostram que produtos adicionados de probióticos ainda estão na vanguarda da inovação no setor de alimentos funcionais (CHAMPAGNE, 2009). No entanto, diversos outros ingredientes, tais como os prebióticos, podem ser aplicados na elaboração de produtos lácteos funcionais, atendendo às exigências do mercado consumidor por alimentos nutritivos e saudáveis. Em relação aos probióticos, a legislação brasileira (ANVISA, 2013) determina que a alegação deve ser definida como " O (indicar a espécie do microrganismo) (probiótico) contribui para o equilíbrio da flora intestinal. Seu consumo deve estar associado a uma alimentação equilibrada e hábitos de vida saudáveis".

3.1. Definição e mercado de alimentos probióticos

Os probióticos são definidos como micro-organismos vivos que, quando administrados em quantidade adequada, conferem efeitos específicos e benéficos à saúde do consumidor (FAO/WHO, 2002). Os principais efeitos atribuídos aos probióticos são regulação da microbiota intestinal e redução do risco de câncer do colón, do nível de colesterol sérico e da ocorrência de diarréias (NAGPAL et al., 2012; FONTANA et al., 2013).

A maioria dos micro-organismos probióticos usados em alimentos é pertencente ao grupo das bactérias acidoláticas (LAB). Apesar de várias cepas de bactérias acidoláticas serem descritas como probióticas, relativamente poucas atendem aos requisitos necessários para produzir os efeitos terapêuticos. Bactérias pertencentes aos gêneros *Lactobacillus* e *Bifidobacterium* são as mais frequentemente empregadas como suplementos probióticos para alimentos. Outros micro-organismos empregados em produtos probióticos pertencem aos gêneros *Enterococcus* (*E. faecium)* e *Saccharomyces* (*S. boulardii*) (OLIVEIRA, 2007). As espécies atualmente consideradas probióticas pela legislação brasileira estão apresentadas na Tabela 1.

Tabela 1. Espécies reconhecidas como probióticas pela legislação brasileira.

Lactobacillus	***Lactococcus***	***Bifidobacterium***	***Enterococcus***
Lactobacillus acidophilus	*Lactococcus lactis*	*Bifidobacterium animalis*	*Enterococcus faecium*
Lactobacillus casei shirota		*Bifidobacterium bifidum*	
Lactobacillus casei var. *defensis*		*Bifidobacterium longum*	
Lactobacillus casei var. *rhamnosus*			
Lactobacillus paracasei			

Adaptado de Anvisa (2013).

Além dos efeitos terapêuticos e nutricionais, os probióticos também têm papel importante na tecnologia de produção dos produtos fermentados devido à sua contribuição ao sabor e aroma dos alimentos, bem como na conservação do produto final, devido ao baixo pH proporcionado pela produção de ácidos e de bacteriocinas (DIVYA et al., 2012).

Os produtos lácteos são os principais carreadores dos probióticos, sendo que os

leites fermentados são as melhores opções do ponto de vista tecnológico (CRUZ et al., 2009a; CRUZ et al., 2009b). No entanto, o crescimento dos probióticos no leite é lento devido à baixa atividade proteolítica, resultando em aumento no tempo de fermentação, o que não é viável do ponto de vista econômico e tecnológico. Por isso, as cepas probióticas são normalmente empregadas como culturas adjuntas e a prática atual é adicionar bactérias da cultura tradicional do iogurte, principalmente *S. thermophilus,* para reduzir o tempo de fermentação (SHIHATA; SHAH, 2002; SHAH, 2007). A Tabela 2 apresenta algumas das culturas probióticas comerciais disponíveis atualmente.

A legislação brasileira, por meio da Comissão de Assessoramento Técnico-Científico em Alimentos Funcionais e Novos Alimentos (CTCAF), recomenda uma porção diária de micro-organismos probióticos viáveis que deve ser ingerida, sendo o mínimo estipulado em 10^8 a 10^9 UFC de micro-organismos por porção diária do produto. Valores menores podem ser aceitos, desde que a empresa comprove sua eficácia. A documentação referente à comprovação de eficácia deve incluir laudo de análise do produto que comprove a quantidade mínima viável do micro-organismo até o final do prazo de validade e teste de resistência da cultura utilizada no produto à acidez gástrica e aos sais biliares (BRASIL, 2008).

Apesar da definição geral de que os probióticos são micro-organismos vivos, inúmeras respostas biológicas foram descritas a partir da administração de células probióticas mortas em várias espécies de mamíferos e aves. As preparações de células mortas também têm sido fracionadas e vários componentes celulares demonstraram capacidade de produzir uma variedade de respostas biológicas. Foi demonstrado que as células probióticas vivas podem influenciar a microbiota gastrointestinal e ter um efeito imunomodulador, enquanto as células mortas e seus componentes podem exercer uma resposta anti-inflamatória. O fato de não ser necessário ter células probióticas vivas para desempenhar o efeito benéfico pode ter um impacto significativo na utilização prática das culturas e na fabricação dos alimentos probióticos. Seria relativamente mais fácil padronizar alimentos fabricados com células mortas, e esses alimentos poderiam ter uma vida de prateleira mais longa, já que a viabilidade dos micro-organismos não seria relevante para o efeito terapêutico. Por fim, a utilização de células mortas permitiria utilizar uma maior gama de micro-organismos considerados como probióticos (ADAMS, 2010).

Tabela 2. Culturas probióticas comerciais.

Micro-organismo	Cepa	Fabricante
Bifidobacterium animalis	Bb-12	Chr. Hansen
Bifidobacterium bifidum	Bb-11	Chr. Hansen
Bifidobacterium infantis	Shirota	Yakult
	Immunitas	Danone
Bifidobacterium lactis	Bb-02	DSM
	Lafti TM	
	B94	
Bifidobacterium longum	BB536	Morinaga Milk Industry
	SBT-2928	Snow Brand Milk Products
Bifidobacterium lactis	DR10	Danisco (Howaru™)
Lactobacillus acidophilus	LA-1/LA-5	Chr. Hansen
	NCFM	Rhodia
	DDS-1	Nebraska Cultures
	SBT-2062	Snow Brand Milk Products
	Lafti L10	DSM Food Specialties
Lactobacillus casei	Shirota	Yakult
	Immunitas	Danone
Lactobacillus fermentum	RC-14	Urex Biotech
Lactobacillus lactis	L1A	Essum AB
Lactobacillus paracasei	CRL 431	Chr. Hansen
Lactobacillus rhamnosus	GG	Valio
	GR-1	Urex Biotech
	LB21	Essum AB
	271	Probi AB
Lactobacillus plantarum	299v	Probi AB
	Lp01	
Lactobacillus reuteri	SD2112/ MM2	Biogaia
Lactobacillus johnsonii	La1	Nestlé

Adaptado de Soccol et al. (2010) e Vasiljevic e Shah (2008).

Em todo o mundo, o consumo de alimentos probióticos tem aumentado consideravelmente nos últimos anos e, junto com outros alimentos e ingredientes funcionais, constituem a fatia de mercado que mais cresce na indústria de alimentos. Estima-se que foram lançados no mercado cerca de 500 novos produtos probióticos na última década (ASHRAF; SHAH, 2011). Na Europa, este setor movimentou em 2008 um total de 1,4 bilhões de euros, liderado principalmente pelos iogurtes e sobremesas, que representam cerca de 72% deste total (SAXELIN, 2008). Uma estimativa recente determinou que o mercado global de produtos probióticos movimenta cerca de 20 bilhões de euros (GASPAR et al., 2013).

3.2. Probióticos: efeitos na microbiota intestinal e na saúde humana

A microbiota do intestino humano, composta por aproximadamente 300-500 espécies, tem um papel crítico na evolução das funções intestinais e na saúde geral do hospedeiro. As células bacterianas excedem as células humanas do hospedeiro e estima-se que a quantidade total de genes das várias espécies existentes no intestino seja de cerca 2-4 milhões, superando o número dos genes humanos em 100 vezes (SAAVEDRA; DATTILO, 2012).

A microbiota intestinal é formada rapidamente após o nascimento e sofre mudanças em sua composição e em suas atividades ao longo de toda a vida, devido à fatores endógenos (por exemplo, idade, estresse, estado imunológico), e a fatores exógenos (por exemplo, dieta, tratamentos médicos). No entanto, esses fatores ainda não são totalmente conhecidos. Análises metagenômicas mostram que, em adultos e em crianças desmamadas a microbiota do cólon é composta principalmente por *Bacteroidetes*, seguido por diversos gêneros pertencentes ao filo *Firmicutes*, tais como *Eubacterium, Ruminococcus* e *Clostridium* e pelo gênero *Bifidobacterium*. Por outro lado, em lactentes o gênero *Bifidobacterium* é predominante e, também estão presentes alguns gêneros da família *Enterobacteriaceae*, tais como *Escherichia, Raoultella* e *Klebsiella.* A presença da microbiota intestinal é de importância fundamental para o desenvolvimento do sistema imunológico da mucosa intestinal e para a manutenção da sua atividade, e também para as inúmeras atividades bioquímicas que realiza (LAPARRA; SANZ, 2010; VINDEROLA et al., 2011a).

As bactérias intestinais podem executar numerosas reações enzimáticas que o hospedeiro não é capaz de catalisar. Esta é a razão pela qual a microbiota é agora considerado como um "órgão dentro de um órgão", com as suas próprias funções: ela modula a expressão de genes envolvidos na fortificação da barreira da mucosa intestinal e na maturação intestinal pós-natal, auxilia na fermentação de substratos não utilizados, convertendo-os em ácidos graxos de cadeia curta, afeta a retirada de energia da dieta e o armazenamento de energia no hospedeiro (HOOPER, 2001).

A microbiota intestinal pode ser modificada com o consumo de probióticos (HAARMAN; KNOL, 2005). Em virtude disso, os probióticos têm sido apontados como o principal fator nutricional capaz de afetar primariamente a fisiologia e as funções do trato gastrointestinal (TGI) (ARVANITOYANNIS; VAN HOUWELINGEN-KOUKALIAROGLOU, 2005).

Durante as últimas décadas, as propriedades nutritivas e terapêuticas das bactérias probióticas têm sido foco de muitos estudos. Entretanto, esses efeitos benéficos conferidos pelos probióticos são cepa-específicos e não espécies ou gêneros-específicos. Ainda, essas propriedades terapêuticas são efetivas somente a partir de um consumo regular dos produtos probióticos. A Tabela 3 apresenta os principais efeitos terapêuticos atribuídos aos probióticos.

A principal atividade resultante da ingestão dos probióticos é a modulação da microbiota intestinal, que ocorre por meio de um mecanismo denominado "exclusão competitiva". Esse mecanismo impede ou reduz a colonização dessa mucosa por micro-organismos potencialmente patogênicos e subsequente produção de toxinas ou invasão das células epiteliais (dependendo do mecanismo de patogenicidade), por meio da competição por sítios de adesão, da competição por nutrientes e/ou da produção de compostos antimicrobianos (KAUR; CHOPRA; SAINI, 2002; GUARNER; MALAGELADA, 2003; TURPIN et al., 2010).

As bactérias acidoláticas geralmente possuem superfície celular com características hidrofóbicas e se agregam com células da mesma espécie ou de outra espécie, o que facilita a adesão ao muco. Essas bactérias também podem expressar as proteínas da superfície celular, que são responsáveis por mediar a adesão ao muco (KMET et al., 1995; LJUNGH; WADSTRÖM, 2006).

Além de aderir e colonizar o epitélio intestinal, os probióticos competem com as bactérias indesejáveis pelos nutrientes disponíveis no nicho ecológico. O hospedeiro fornece as quantidades de nutrientes que as bactérias probióticas necessitam e estas iniciam ativamente as suas necessidades. Essa relação simbiótica impede a produção excessiva de nutrientes, a qual favoreceria o estabelecimento de micro-organismos com potencial patogênico (KOPP-HOOLIHAN, 2001; GUARNER; MALAGELADA, 2003).

Os probióticos podem ainda impedir a multiplicação de seus competidores, pela produção de compostos antimicrobianos. As substâncias antimicrobianas produzidas pelos micro-organismos probióticos, os ácidos orgânicos (acético, lático, fórmico) e as substâncias antimicrobianas (ácidos graxos, peróxido de hidrogênio, dióxido de carbono, etanol, diacetil, acetaldeído, D-leucina, enzimas bacteriolíticas, bacteriocinas, antifúngicos e antibióticos) são responsáveis pelos efeitos bactericida ou bacteriostático contra espécies patogênicas (REIS et al., 2012).

Tabela 3. Propriedades terapêuticas e benéficas atribuídas aos probióticos.

Efeitos benéficos	Possíveis causas e mecanismos
Alívio da intolerância à lactose	Redução de lactose no produto e maior disponibilidade de β-galactosidase no trato gastrointestinal
Ação contra patógenos entéricos	Exclusão competitiva, produção de compostos antimicrobianos e prevenção da adesão de patógenos
Controle e prevenção de alergias	Barreira imunológica, modulação do sistema imune
Efeito anticarcinogênico e antimutagênico	Conversão de compostos carcinogênicos em compostos inócuos, degradação de pré-carcinógenos, redução de enzimas promotoras de processos cancerígenos, normalização da permeabilidade intestinal e estimulação do sistema imune
Ação hipocolesterolêmica	Produção de inibidores da síntese de colesterol, uso de colesterol por assimilação e precipitação com sais biliares deconjugados
Modulação imunológica	Melhora a formação de macrófagos, estimula a produção das células supressoras e de γ-interferon, diminui produção de citocinas pró-inflamatórias

Adaptado de Vasiljevic; Shah (2008).

Recentemente, a produção de vitaminas pelas bactérias acidoláticas ganhou atenção da comunidade científica (SMID; LACROIX, 2013). Embora as bactérias acidoláticas sejam exigentes nutricionalmente, certas cepas têm capacidade de sintetizar vitaminas do grupo B (tiamina, ácido fólico e a riboflavina) e vitamina K. Além de melhorar o perfil nutricional dos alimentos, a utilização de culturas *starter* selecionadas para a fermentação também pode levar os micro-organismos para o intestino, onde eles irão, potencialmente, sintetizar as vitaminas (O´CONNOR et al., 2005; LEBLANC et al., 2010). Além disso, a utilização de cepas probióticas produtoras de folato podem conferir proteção eficaz contra inflamação e câncer, porque além de exercerem os efeitos probióticos, previnem contra a deficiência de folato, que está associada às alterações no epitélio do cólon, que normalmente ocorrem antes do desenvolvimento do câncer (ROSSI; AMARETTI; RAIMONDI, 2011).

3.3. Fatores que afetam a sobrevivência dos probióticos

Para que desempenhem seus efeitos benéficos no hospedeiro, os probióticos devem ser capazes de reter sua viabilidade em três etapas críticas: i) fabricação do

produto, ii) durante todo o período de estocagem do produto e iii) durante a passagem pelo TGI após o consumo (FIGUEROA-GONZALEZ et al., 2011).

3.3.1. Sobrevivência nos alimentos

Dentre os fatores que afetam a viabilidade do probiótico no produto estão: o conteúdo de sólidos totais do leite, o tipo de matriz alimentícia, a presença de conservantes e micro-organismos contaminantes, a disponibilidade de nutrientes no leite, a concentração de açúcar e de inóculo, interações entre os micro-organismos presentes, as temperaturas de incubação e de armazenamento, a pós-acidificação e o conteúdo de peróxido de hidrogênio devido ao metabolismo microbiano durante a estocagem, a concentração de oxigênio no produto e a permeabilidade de oxigênio pela embalagem (VINDEROLA et al., 2002; DONKOR et al., 2006; OLSON; ARYANA, 2008; SAINT-EVE et al., 2008; ALMEIDA; TAMIME; OLIVEIRA, 2009; SACCARO et al., 2009; RAMCHANDRAN; SHAH, 2010; MOHAMMADI; SOHRABVANDI; MORTAZAVIAN, 2012).

O aumento da viabilidade e sobrevivência da cepa probiótica no alimento funcional pode ser obtida por seleção de cultura apropriada (LOURENS-HATTINGH; VILJOEN, 2001; TUOMOLA et al., 2001), microencapsulação (HEIDEBACH; FORST; KULOZIK, 2012; ZIAR; GÉRARD; RIAZI, 2012), suplementação do leite com ingredientes de diferentes origens (CRUZ et al., 2012; ESPIRITO SANTO et al., 2012; BEHESHTIPOUR et al., 2013) ou o uso de promotores de crescimento, tais como prebióticos (OLIVEIRA et al., 2009; OLIVEIRA et al., 2011).

Após o consumo, os probióticos precisam sobreviver às condições adversas do TGI, além de resitir a outros componentes que possam estar presentes nesse local, dependendo do indivíduo, tais como a presença de medicamentos.

3.3.2. Sobrevivência durante a passagem pelo trato gastrointestinal

A fim de desempenhar os seus efeitos benéficos, as cepas probióticas devem sobreviver às condições adversas do TGI, tolerando o ácido, os sais biliares e enzimas gástricas e entéricas, e depois aderir e colonizar no epitélio intestinal (VINDEROLA et al., 2011). Dentre os fatores prejudiciais para a viabilidade dos probióticos no estômago

estão o baixo pH e ação antimicrobiana de pepsina. O pH do estômago varia, geralmente, de 2,5 a 3,5, mas pode ser tão baixo quanto 1,0 ou 2,0, com taxas mais elevadas de secreção de suco gástrico (MARAGKOUDAKIS et al., 2006). Após passarem pelo estômago, os probióticos ingeridos devem sobreviver à passagem pelo intestino delgado, onde eles são expostos à pancreatina, sais biliares e um valor de pH de cerca de 8,0 (RANADHEERA et al., 2012).

Várias abordagens têm sido investigadas para melhorar a sobrevivência dos probióticos às condições do TGI, incluindo a proteção física (microencapsulação) (DIANAWATI; MISHRA; SHAH, 2013; GEBARA et al., 2013), utilização de ingredientes alimentares, tais como concentrado de proteína de soro (WPC) e inulina (BURITI; CASTRO; SAAD, 2010) e utilização de uma matriz alimentar mais apropriada (FORSSTEN; SINDELAR; OUWEHAND, 2011; RANADHEERA et al., 2012).

Atualmente, é bem estabelecido o fato de que as matrizes alimentares desempenham um papel importante na proteção os probióticos durante o armazenamento dos alimentos e trânsito pelo TGI. Portanto, no desenvolvimento de alimentos probióticos é extremamente importante selecionar veículos adequados para o carreamento de probióticos, para garantir que eles superem as barreiras físicas e bioquímicas encontradas no TGI (WANG et al., 2009; MARTINEZ et al., 2011).

A resistência aos sucos gástrico e entérico simulados está entre os ensaios *in vitro* que são frequentemente sugeridos para a avaliação do potencial probiótico de uma cepa (BURITI; CASTRO; SAAD, 2010; GBASSI et al., 2011; BEDANI; ROSSI; SAAD, 2013). Esta avaliação pode ajudar na seleção uma matriz alimentícia adequada e contribuir para a sobrevivência e eficácia do probiótico no TGI (SCHILLINGER; GUIGAS; HOLZAPFEL, 2005; BURITI; CASTRO; SAAD, 2010).

Além das condições adversas do TGI, a presença de medicamentos nesse local pode também representar um fator de estresse para os micro-organismos probióticos, suprimindo ou reduzindo seu efeito terapêutico (TODOROV et al., 2008).

Várias pessoas que consomem alimentos probióticos podem estar, simultaneamente, em tratamentos medicamentosos. Portanto, os indivíduos que ingerem medicamentos devem estar cientes de que essas substâncias podem reduzir os efeitos benéficos das bactérias probióticas consumidas. Apesar da importância desta interação, não há muitas pesquisas que se dedicam a avaliar o efeito de um largo espectro das drogas sobre o crescimento de cepas probióticas comerciais usadas nos produtos fermentados.

O efeito negativo dos medicamentos sobre os probióticos é mais preocupante no caso de drogas para controle de doenças crônicas, que podem acumular no TGI dos pacientes devido ao consumo contínuo e prolongado. O efeito de medicamentos tomados livremente (sem prescrição) pela população de todas as faixas etárias, como os analgésicos, também deve ser levado em consideração. Conhecendo as interações entre esses tipos de drogas e os probióticos, farmacêuticos e médicos poderão recomendar melhor o tipo de analgésico que pode ser utilizado simultaneamente com os probióticos, em cada caso específico, ou ainda, indicar qual cepa probiótica não é afetada pelo medicamento, para evitar falha do efeito terapêutico desses micro-organismos.

3.4. Adesão dos probióticos às células intestinais

A capacidade de aderir às superfícies das mucosas do intestino e a subsequente colonização a longo ou a curto prazo tem sido um dos critérios mais comuns para a seleção de cepas probióticas (COLLADO et al., 2009). Para desempenhar os efeitos benéficos no intestino grosso, tais como a exclusão competitiva de patogênos do epitélio intestinal ou a imunorregulação, o probiótico deve ser capaz de, pelo menos temporariamente, colonizar a mucosa intestinal. Os mecanismos de adesão não estão completamente compreendidos, no entanto, proteínas presentes na superfície das células bacterianas e propriedades de ligação às células intestinais foram identificados e caracterizados em cepas probióticas (VELEZ; DE KEERSMAECKER; VANDERLEYDEN, 2007; SANCHEZ; BRESSOLLIER; URDACI, 2008). Apesar de novas e sofisticadas metodologias serem criadas a cada ano, a capacidade de adesão bacteriana é o ensaio mais utilizado nos estudos *in vitro* (JENSEN et al., 2012). As células da linhagem Caco-2 é um dos modelos mais utilizados no teste de adesão *in vitro* às células intestinais (HUANG; ADAMS, 2003). Essa linhagem celular foi originalmente isolada a partir de adenocarcinoma do cólon humano (PINTO et al., 1983).

As células epiteliais do intestino estão cobertos por uma camada protetora de muco, o que oferece pontos de fixação para bactérias no intestino. Proteínas extracelulares de ligação ao muco, tais como MUB em *L. reuteri* 1063 (ROOS; JONSSON, 2002) e a adesina manose-específica (MSA) de *L. plantarum* WCFS1 (PRETZER et al., 2005) são exemplos importantes de fatores de adesão. Muitas outras LAB tem domínios semelhantes, indicando a possível presença de proteínas de ligação

ao muco (BOEKHORST et al., 2006). Além disso, outras moléculas da superfície celular, tais como as proteínas da camada S, o ácido lipoteicóico e os exopolissacarídeos, também contribuem para a adesão específica e/ou não específica às células epiteliais do hospedeiro (LEBEER; VANDERLEYDEN; DE KEERSMAECKER, 2008).

A capacidade de adesão dos probióticos às células intestinais pode ser influenciada pelos ingredientes e pelo tipo de matriz alimentícia carreadora desses micro-organismos. Portanto, é importante avaliar as interações entre o alimento e as superfícies bacterianas, porque eles podem afetar as propriedades físico-químicas da superfícies das células bacterianas e, consequentemente, a sua adesão à mucosa intestinal. Apesar disso, poucos estudos foram feitos para verificar o efeito do alimento ou de componentes específicos sobre as propriedades de aderência de células bacterianas (DEEPIKA; RASTALL; CHARALAMPOPOULOS, 2011).

Os resultados *in vitro* para a capacidade de aderência bacteriana às células epiteliais são difíceis de extrapolar para a situação do TGI humano, onde o sistemas de defesa do hospedeiro, a competição com a microbiota, a descamação da mucosa e o fluxo peristáltico podem modificar a adesão bacteriana (LEBEER; VANDERLEYDEN; DE KEERSMAECKER, 2008). No entanto, os ensaios *in vitro* são essenciais para compreender os mecanismos de adesão e fornecer informações importantes sobre as diferenças entre espécies e cepas bacterianas estudadas (JENSEN et al., 2012).

3.5. Uso de prebióticos

Os produtos lácteos representam um grande potencial de investimento para a indústria de alimentos que pode ser explorado por meio do desenvolvimento de ingredientes, processos e produtos inovadores. A suplementação do leite utilizado para a fabricação de leites fermentados probióticos com ingredientes prebióticos representa a possibilidade de, simultaneamente, incrementar o valor nutritivo do produto, uma vez que estimula o crescimento e viabilidade dos probióticos durante a estocagem e é fonte adicional de nutrientes, tais como minerais, vitaminas e ácidos graxos essenciais, além de atender a demanda do mercado consumidor por produtos inovadores (MOHAMMADI; MORTAZAVIAN, 2011). O prebiótico é, portanto, um ingrediente que resulta em mudanças específicas na composição e/ou atividade da microbiota gastrointestinal, conferindo assim benefícios sobre a saúde do hospedeiro

(ROBERFROID et al., 2010). De acordo com a legislação brasileira, para o alimento prebiótico possuir a alegação de produto funcional ele deve fornecer no mínimo 3 g de inulina ou frutooligossacarídeo (FOS) se for sólido ou 1,5 g de inulina ou FOS se for líquido na porção diária (ANVISA, 2013).

Os alimentos que combinam prebióticos e probióticos são denominados simbióticos. Vários estudos têm mostrado que o crescimento dos probióticos pode ser aumentado em iogurtes ou produtos lácteos fermentados na presença de prebióticos como inulina, polidextrose e oligofrutose (OLIVEIRA et al., 2009; RANADHEERA; BAINES; ADAMS, 2010), permitindo que a população viável esteja acima do nível exigido pela legislação (10^7 - 10^9 UCF/mL do produto).

A inulina é um dos ingredientes prebióticos mais conhecido e estudado atualmente (SLAVIN, 2013), cuja composição contém uma mistura de polímeros de D-frutose com grau de polimerização de 2 a 60. Quimicamente consiste em moléculas de sacarose, nas quais uma, duas ou três unidades de frutose são adicionadas por ligações do tipo β-(2→1) à molécula de frutose da sacarose (OLIVEIRA; PENNA, 2009). A inulina é oficialmente reconhecida como ingrediente alimentício natural e é classificada como fibra alimentar pela legislação brasileira e em alguns países europeus (ROBERFROID, 2002; BRASIL, 2008), e apresenta baixo valor calórico, uma vez que as enzimas do TGI humano não são capazes de hidrolisar as ligações que unem as moléculas de frutose de sua estrutura (OLIVEIRA; PENNA, 2009).

3.5.1. Uso de farinhas vegetais na elaboração de leites fermentados probióticos

Nos últimos anos tem sido estudado o potencial da adição de fibras e farinhas de frutas e de outros vegetais nas características do leite fermentado. A fortificação com fibras de banana, maçã e maracujá melhorou a viabilidade de probióticos e o perfil de ácidos graxos em leite fermentado (ESPIRITO SANTO et al., 2012). Um estudo demonstrou que as farinhas de grão de bico, de lentilha, de ervilha e de soja melhoraram a taxa de acidificação das culturas probióticas *Lactobacilus rhamnosus* AD200 e *Lactobacilus acidophilus* AD200 durante a fermentação de iogurtes, sendo que os melhores resultados foram observados para as farinhas de ervilha e de soja (ZARE et al., 2012). A adição de 4% de farinha de lentilha ao leite também mostrou ser capaz de aumentar a viabilidade das populações de *L. acidophilus* B-4495 e *B. animalis* 41405 em cerca de 1 log em relação ao leite fermentado controle (AGIL et al., 2013).

Dentre os vegetais que podem ser explorados como ingredientes para suplementação do leite estão a banana, maçã, uva e quinoa. A banana (*Musa* sp., Musaceae) é altamente nutritiva e mais facilmente digerida do que outras frutas. O tempo de digestão da banana (105 min) é menor do que o da maçã (210 min). A banana é popular devido ao seu aroma, textura e facilidade para descascar e comer, além de ser rica em potássio e cálcio e apresentar baixo conteúdo de sódio. O sub-produto dessa fruta contém cerca de 43-49 g de fibra dietética total, 1 g de inulina, 6 g de frutooligossacarídeo e 10-20 g de pectina por 100 g de matéria seca, além de quantidades significativas de α-linolénico (ALA), aminoácidos essenciais e micronutrientes, tais como magnésio, potássio, fósforo e cálcio (EMAGA et al., 2008; MOHAPATRA; MISHRA; SUTAR, 2010).

A maçã (*Malus* sp., Rosaceae) constitui uma parte significativa da dieta humana, sendo reconhecida como uma das principais fontes dietéticas de antioxidantes, principalmente na forma de compostos fenólicos, tais como os flavonóides e ácidos fenólicos, e exibe elevada capacidade antioxidante (HENRÍQUEZ et al., 2013). Foi relatado que 100 g de matéria seca do sub-produto de maçã pode conter cerca de 46 g de fibra insolúvel, 14 g de fibra solúvel (CHEN et al., 1988), enquanto que a pectina e frutooligossacarídeo contribuem para 8-12 e 4,9 % de fibra dietética total (DF), respectivamente (NAWIRSKA; KWAŚNIEWSKA, 2005).

A uva (*Vitis* vinifera) é uma das culturas de frutas mais cultivadas do mundo, com mais de 60 milhões de toneladas produzidas anualmente (RONDEAU et al., 2013). Entre as frutas, as uvas constituem uma das principais fontes de compostos fenólicos (YILDIRIM et al., 2005) e de antioxidante de baixo custo (GARRIDO et al., 2011). Além disso, a casca da uva tem potencial antimicrobiano (KATALINIĆ et al., 2010). A utilização do sub-produto da uva combina, portanto, os benefícios de uma maior ingestão de fibra dietética total com seus antioxidantes naturais.

A quinoa (*Chenopodium quinoa* Willd) é um alimento básico de civilizações antigas, caracterizada por ser uma planta tolerante a estresse, com ecotipos crescendo bem em solos com altas salinidades (RUIZ-CARRASCO et al., 2011), em altitudes elevadas e solos pobres, com chuvas limitadas ou sob irrigação extremamente baixa (MARTÍNEZ et al., 2009; FUENTES; BHARGAVA, 2011). Este grão é altamente nutritivo e tradicionalmente cultivado no planalto andino do Peru, Bolívia, Equador, Chile, Argentina e Colômbia (ABUGOCH JAMES, 2009). Um estudo analisou seis diferentes tipos de genótipos de quinoa encontrados no Chile e relatou que 100 g de

matéria seca de quinoa oferece de 11 a 16 g de proteína, enquanto o conteúdo de fibra alimentar total varia de 8-12 g. Também se observou um equilíbrio adequado de aminoácidos essenciais e a sacarose foi o açúcar dominante em todos os genótipos avaliados (MIRANDA et al., 2012).

Portanto, a avaliação da adição de farinhas obtidas dessas fontes vegetais ao leite fermentado probiótico poderá contribuir para a formulação de novos produtos, saudáveis e com apelo funcional, com níveis elevados de fibras dietéticas, minerais, vitaminas e outros componentes bioativos.

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Capítulo 2

Este trabalho será submetido ao *International Dairy Journal*

INFLUENCE OF THE COMBINATION OF PROBIOTIC CULTURES DURING FERMENTATION AND STORAGE OF FERMENTED MILK

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ABSTRACT

The objective of this study was to evaluate the influence of different combinations of cultures (*Lactobacillus acidophilus* - La and *Bifidobacterium animalis* subsp. *lactis* - Bb as pure cultures or in co-culture with *Streptococcus thermophilus* - St) on fermented milk during fermentation (including changes to the acidification profile, organic acid production and lactose consumption) and during 28-day storage (in terms of bacteria viability, syneresis, sensory properties, organic acid content and viability under simulated *in vitro* gastrointestinal conditions) at 4 °C. La culture was showed the lowest acidification rate (V_{max}) values, while the pure St culture showed the highest V_{max} values. During fermentation, Bb produced the largest amount of acetic acid, and only La was able to metabolize citric acid. Syneresis decreased during storage for all treatments. Populations of *S. thermophilus* and *B. animalis* subsp. *lactis* remained stable during the storage period in all treatments, while the population of *L. acidophilus* decreased over time only in the case of the La treatment. The simulation of the *in vitro* survival in the gastrointestinal tract indicated that bifidobacteria possesses a greater tolerance to acid and bile than the lactobacilli strain. The La treatment resulted in lower scores for all attributes in both periods of sensory analysis. When lactic acid was present in smaller quantities and citric acid was present in larger amounts, the scores regarding flavor and overall acceptability attributes were higher. Depending on the combination of microorganisms used in fermented milk manufacturing, it had positive or negative impacts on the product's characteristics.

Keywords: lactic acid bacteria, probiotics, kinetic acidification parameters, sensory properties, organic acids, gastrointestinal tract.

1. INTRODUCTION

Fermented milk is the result of milk acidification through the metabolic activity of lactic acid bacteria (LAB), which causes important physicochemical, sensory and microbiological changes in fermented milk products. In order to manufacture fermented milk, the traditional yogurt culture, consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which contribute to the appropriate sensory characteristics of yogurt, can be used. However, in recent years, the trend has changed and probiotic bacteria have been added to fermented milk more frequently. These bacteria, unlike those of the traditional culture, promote beneficial health effects for the consumer, if consumed regularly and in adequate amounts (FAO/WHO, 2002). In order to exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form. However, no general agreement has been reached regarding the recommended levels, and suggested levels have ranged from 10^6 CFU/mL to over 10^7 and 10^8 CFU/mL (VASILJEVIC; SHAH, 2008).

Lactobacillus acidophilus and *Bifidobacterium animalis* subsp. *lactis* are the lactic acid bacteria that are most frequently used as probiotics. Several studies have reported the loss of viability of probiotic microorganisms in fermented milk during production and storage. Among the factors affecting the viability of such bacteria are the solids content of the milk (ALMEIDA; TAMIME; OLIVEIRA, 2009), the type of food matrix (ESPÍRITO-SANTO et al., 2011), the presence of preservatives and other microorganisms (VINDEROLA et al., 2002; VINDEROLA; MOCCHIUTTI; REINHEIMER, 2002), inoculum concentration (OLSON; ARYANA, 2008), incubation temperature (ØSTLIE; TREIMO; NARVHUS, 2005), and an increase in acidity and temperature during storage (DONKOR et al., 2006).

Probiotics exhibit low proteolytic activity and, therefore, develop slowly in milk, resulting in a long fermentation time. For this reason, they are usually combined with microorganisms of the yogurt culture, which facilitate their development into the milk through their high proteolytic activity. Thus, the use of these probiotic cultures allows dairy processors to produce fermented dairy products with the desired technological characteristics, as well as with potential nutritional and health benefits (CANDY et al., 2008). However, microbial interactions among these cultures, whether beneficial (protocooperation) or unfavorable (antagonism) can occur. As a consequence,

undesirable changes in the composition of the bacterial population may result during manufacturing and cold storage of fermented milk (SACCARO et al., 2009).

To avoid loss of probiotic viability, some producers and researches have removed *L. delbrueckii* subsp. *bulgaricus* from starter cultures containing probiotics, because this species is detrimental to probiotic survivability due to its acidification activity during product storage. Currently, probiotics are largely used in combination with other microorganisms, such as *Streptococcus thermophilus*. For this reason, it is important to study the interactions between the species of probiotics currently used in the manufacturing of fermented milk, such as *L. acidophilus* and *B. animalis*, along with *S. thermophilus*.

The sensory profile of fermented milk is also directly influenced by the metabolic activity of the bacteria, which interact strongly with the components of the media to convert certain metabolic products during the growth, particularly organic acids (SERRA et al., 2009). Furthermore, the species of bacteria used in fermented milk manufacturing has an impact on the physical characteristics of the product. Clot formation is the most important functional property of dairy products, and the rheological characteristics of their gel are affected by the starter culture selected. When the fermentation process does not occur properly, the formation of the protein network will be disorganized and the final product may have technological defects, such as low water holding capacity and syneresis (LUCEY, 2004).

The possible interactions among the strains selected to manufacture a dairy product should be taken into account to select the best combination(s) and to optimize their technological performance in the process and their survival in the products during cold storage (VINDEROLA; MOCCHIUTTI; REINHEIMER, 2002). Therefore, the objective of this study was to evaluate the changes in fermented milk with different culture combinations during fermentation,including changes to the acidification profile, organic acid production and lactose consumption, and 28-day storage in terms of bacteria viability, syneresis, sensory properties, organic acid content and viability under simulated *in vitro* gastrointestinal conditions at 4 °C.

2. MATERIAL AND METHODS

2.1. Inoculum preparation

Three commercial starter freeze-dried strains were used in this study – specifically, *S. thermophilus* TA040 (St) (Danisco, Sassenage, France), *B. animalis* subsp. *lactis* BB-12 (Bb) and *L. acidophilus* La-5 (La) (Chr. Hansen, Valinhos, Brazil). Pure freeze-dried strains in amounts recommended by the manufacturer were individually suspended in 50 mL of sterilized skim milk (10% w/v), and were then activated at 42 °C for 30 min before use. One mL of each activated culture was inoculated in 250 mL of milk according to the treatment, which allowed for initial counts of approximately 6 log CFU/mL after milk inoculation.

2.2. Fermentation and kinetic parameters

Skim powder milk (Nestlé, Araçatuba, Brazil) was reconstituted to 13% (w/w) in distilled water. Reconstituted milk was thermally treated at 90 °C for 10 min in a water bath and distributed in 500-mL sterile bottles inside a laminar flow chamber, and then stored at 4 °C for 24 h before use. On the day of fermentation, the milk bottles were warmed to 42 °C and the cultures were added (0.4%) according to the treatment. In addition, 40-mL aliquots of the inoculated milk were aseptically distributed in 50-mL sterile flasks. One flask was prepared for each sampling time and it was used for all analyses. The pH value, organic acids and lactose contents were determined in the incubated milk before fermentation, at pH values of 6.0, 5.5, 5.0 and 4.6 (end of fermentation).

Five treatments were conducted in three separate independent trials: St (composed of *S. thermophilus* TA040), La (composed of *L. acidophilus* La-5), Bb (composed of *B. animalis* subsp. *lactis* BB-12), StBb (composed of *S. thermophilus* TA040 + *B. animalis* subsp. *lactis* BB-12), and StLa (composed of *S. thermophilus* TA040 + *L. acidophilus* La-5). After inoculation, flask samples were transferred to a water bath that was connected to a CINAC (Cynetique d´acidificacion, Alliance Instruments, Frepillon, France) system that allows for the continuous measurement and recording of pH values, as well as the evaluation of the acidification kinetics throughout the run. Batch fermentations were performed at 42 °C up to a pH of 4.6, which was

selected as the condition for stopping fermentation. Afterward, fermented milk was transferred to an ice bath and cooled down to 15 °C. The clot was broken down using a stainless steel perforated disk with up and down movements for approximately 1 min. The product was put in 80-mL sterile plastic cups, and stored at 4 °C for 28 days.

From the data collected during fermentation, the acidification rate (V_{max}) was calculated as the time variation of the pH (dpH/dt) and expressed as 10^{-3} pH units/min. During the incubation period, the following kinetic parameters were also calculated: (i) t_{max} (h), time at which V_{max} was reached; (ii) $t_{pH5.0}$ (h), the time required to reach pH 5.0; and (iii) $t_{pH4.6}$ (h), the time required to reach pH 4.6 (i.e., to complete fermentation) (ALMEIDA; TAMIME; OLIVEIRA, 2009).

2.3. Post-acidification and spontaneous whey separation

Fermented milk post-acidification was determined 1 day after the fermentation was complete and after 14 and 28 day-storage at 4 °C through the use of pH measurements (performed in triplicate) using a pHmeter model PG1800 (Gehaka, São Paulo, Brazil). The susceptibility of fermented milk to syneresis was determined using a drainage method after 1, 14 and 28 days of storage. The test was performed at 6 °C in triplicate. One hundred mL of fermented milk was transferred into a funnel fitted with a 120 mesh stainless steel screen. The volume of the whey collected over 2 h was measured in a 50-mL graduated cylinder. The results were expressed in % of whey released (HASSAN et al., 1996).

2.4. Microbiological analyses

Bacterial counts of each treatment were carried out in duplicate after 1, 14 and 28 days of storage. *S. thermophilus* colonies were enumerated in M17 agar (Himedia, Mumbai, India), whereas those of *L. acidophilus* were carried out in MRS agar (Acumedia, Lansing, USA) with a bile solution added (Sigma, St. Louis, USA), and those of *B. animalis* subsp. *lactis* in MRS agar (Acumedia, Michigan, USA) with 0.2 and 0.3 % lithium chloride and sodium propionate (Sigma), respectively. The M17, MRS-bile and MRS-LP media were prepared according to IDF (1997), IDF (1995), and Vinderola and Reinheimer (1999), respectively. Plates of *S. thermophilus* and *L.*

acidophilus were incubated under aerobic conditions at 37 °C for 72 h. Plates of *B. animalis* subsp. *lactis* were incubated under anaerobic conditions provided by Anaerobac (Probac, São Paulo, Brazil). Cell concentration was expressed as log CFU/mL of fermented milk.

Microbiological safety of fermented milk samples was examined before the sensory evaluation to evaluate the yeasts and molds using Yeast and Mold Compact Dry YM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) incubated at 25 °C, as well as *Escherichia coli* and total coliforms using Compact Dry CF and EC (Nissui Pharmaceutical Co) incubated at 45 °C and 35 °C, respectively.

2.5. Survival of probiotics under simulated gastrointestinal conditions

The survival of probiotics under simulated gastric and enteric conditions was evaluated in duplicate after 1, 14 and 28 days of refrigerated storage. In the gastric phase, fermented milk samples were diluted in a 0.5% NaCl solution, and 10 mL was transferred to 3 sterile flasks. The pH was adjusted to 1.4–1.9 with 1 N HCl, and pepsin (Sigma-Aldrich, St. Louis, USA) and lipase (Aldrich Chemical Company, Milwaukee, USA) solutions were added to samples to reach a concentration of 3 g/L and of 0.9 mg/L, respectively. Flasks were incubated at 37 °C, with an agitation rate of approximately 150 rpm (Metabolic Water Bath Dubnoff MA-095, Marconi, Piracicaba, Brazil) for 2 h. In the enteric phase I, the pH of the samples was increased to 4.3–5.2 using an alkaline solution containing bile (Sigma-Aldrich) and pancreatin (Sigma-Aldrich) at concentrations of 10 g/L and 1 g/L, respectively, followed by incubation at 37 °C for 2 h under agitation. In the enteric phase II, the pH was increased to 6.7–7.5 using the same alkaline solution containing bile and pancreatin (the same concentrations were maintained), and samples were incubated again at 37 °C for 2 h under agitation (BURITI; CASTRO; SAAD, 2010). Enumeration of probiotics was performed in aliquots (1 mL) collected from duplicate samples after 30 min, 120 min, 240 minand 360 min. The media employed were the same as those described previously.

2.6. Sensory analyses

Sensory analysis was performed at the Sensory Analysis Laboratory, part of the Department of Food Technology and Engineering, within the Instituto de Biociências,

Letras e Ciências Exatas at São Paulo State University after 14 and 28 days of refrigerated storage, using individual booths with white light. This study was approved by the Research Ethics Committee of the Institute (Supplementary material 1) (CEP 065/09). An independent trial was conducted to sensory analyses.

In each session, five samples (20 mL) packed in plastic white cups and identified with random three-digit codes were served to the untrained panelists (60, different panelists between days of analysis) in a randomized order. The panelists were students and members of the staff of the institute. Between samples, the panelists were instructed to rinse their palates with crackers and tap water.

The attributes of overall appearance, consistency, odor, flavor and overall acceptability were scored according to a hedonic scale (1 = dislike extremely, 3 = dislike moderately, 5 = neither like nor dislike, 7 = like moderately, and 9=like extremely) (Supplementary material 2).

2.7. Chemical analyses

Lactose was measured in duplicate using Dionex equipment, model ICS-5000 DC (Thermo Scientific, Sunnyvale, USA). Samples were diluted in Milli-Q water and filtered through membrane filters with 0.22-µm pore diameters (Millipore^{TM}) in a Dionex vial. Separation was achieved using a Carbopac PA100 column (250 x 4 mm i. d.) at 40 °C. The injection volume was 15 µL. The solvents used were water (solvent A), 500 mM NaOH (solvent B), and 200 mM NaOH/200 mM sodium acetate (solvent C). The flow rate was 1.0 mL/min. The gradient elution was as follows: 0 min, 50% for A and C; 9 min, 5% for A and C and 90% for B; 10 min, 50% for A and C.

Organic acids were measured in duplicate according to Oliveira et al. (2012) and Zeppa; Conterno and Gerbi (2001). In brief, milk or fermented milk samples were mixed with 80 mL of 15.5-M nitric acid and then diluted with 1.0 mL of the mobile phase of 0.013-M sulphuric acid (Merck, Darmstadt, Germany). The resulting mixture was centrifuged at 14,000 x *g* for 30 min using a centrifuge (Beckman Coulter, Avanti J26XP, Brea, USA) for the removal of proteins. The supernatant was filtered through a 0.20-mm membrane filter into an HPLC vial. The separation of organic acids was achieved using a Perkin Elmer HPLC (PerkinElmer Inc., Walthan, USA) fitted with an Aminex HPX-87H, 300 x 7.8-mm ion-exchange column (Biorad Life Science Group,

Hercules, USA) and a guard column maintained at 65 °C. The mobile phase was 0.013 M H_2S0_4 with a flow rate of 0.8 mL/min.

2.8. Statistical analyses

Results were analyzed using two-way ANOVA, which was performed with the STAT Software version 2.0. Mean values were compared using the Tukey test at $P \leq 0.05$. Pearson correlation analysis was performed between the amount of organic acids and the sensory acceptability of the samples using the Minitab 16 software (Minitab Inc., Pennsylvania, USA), at the level $P \leq 0.05$ of significance.

3. RESULTS AND DISCUSSION

3.1. Evaluation during fermentation

3.1.1. Lactose and organic acids

There was a reduction in the lactose content during fermentation (data not shown), as expected, and samples of the treatments fermented by mixed cultures had lower levels of lactose (25.98 g/L for StLa and 25.94 g/L for StBb) at the end of fermentation (pH 4.6). This result indicates that *S. thermophilus* stimulated the probiotic bacteria, which resulted in increased hydrolysis of lactose.

Organic acids play an important role as natural preservatives in fermented dairy products and also contribute to sensory properties (REIS et al., 2012). The presence of these compounds in fermented milk indicates the existence of metabolic activity of microorganisms over affecting lactose (ØSTLIE; TREIMO; NARVHUS, 2005).

For the production of good-quality fermented milk (acidity and firmness of the clot), the lactate concentration should be around 8000 mg/L (NARVHUS et al., 1998). The amount of lactic acid produced at the end of fermentation depended on the type of culture used, and it ranged from 5072 to 8495 mg/L (Figure 1a). The homofermentative bacteria *S. thermophilus* and *L. acidophilus* in pure culture or in co-culture produced the largest amount of lactic acid using the Embden-Meyerhof-Parnas pathway (glycolysis), while the pure culture of *B. animalis* subsp. *lactis* resulted in the lowest lactic acid content. Similar results were observed by Oliveira et al. (2012). Acetic acid

concentration ranged from 44.82 to 4189 mg/L (Figure 1b), and not surprisingly, the samples with high acetic acid content at the end of fermentation were those containing the strain *B. animalis* subsp. *lactis*. The pure St culture produced more lactic acid (+ 6.56%) than the StBb co-culture. On the other hand, the concentration of acetic acid in the StBb co-culture was smaller (- 82.5%) than that produced by the pure culture Bb, due to the inhibition of heterofermentative characteristics of *B. animalis* subsp. *lactis* by *S. thermophilus*. This phenomenon contributes to the development of products with better sensory quality, because high amounts of acetic acid result in dairy-flavored vinegar, making the product unappealing to the consumer (RODRIGUES et al., 2011).

B. animalis subsp. *lactis* is an heterofermentative strain that ferments lactose through a specific route called bifidus pathway, which is characterized by the presence of the enzyme fructose 6-phosphate phosphoketolase. Theoretically, the fermentation of two glucose molecules leads to 3 mols of acetic acid and 2 mols of lactic acid (ØSTLIE; TREIMO; NARVHUS, 2005; OLIVEIRA et al., 2012). However, a study demonstrated that the fermentation temperature used may alter the ratio between lactic and acetic acids (ØSTLIE; TREIMO; NARVHUS, 2005). In products fermented at 30 to 37 °C the ratio between lactate and acetate was 3:2, but after incubation at 45 °C, the rate was 1.7:2. In this study, the incubation temperature was 42 °C and, therefore, the molar ratio between acetate and lactate was of 2.35:2, lower than the theoretical one. The production of acetic acid in smaller amounts is important from a sensory standpoint (DONKOR et al., 2007). The use of a fermentation temperature that is higher than the ideal (37 °C) for *B. animalis* subsp. *lactis* may have reduced the activity of the enzyme aldehyde dehydrogenase, resulting in less formation of acetic acid. The ratio of lactic acid to acetic acid was lower (0.36:2) in the StBb co-culture, since *S. thermophilus* produces acetate in lower amounts when compared to bifidobacteria.

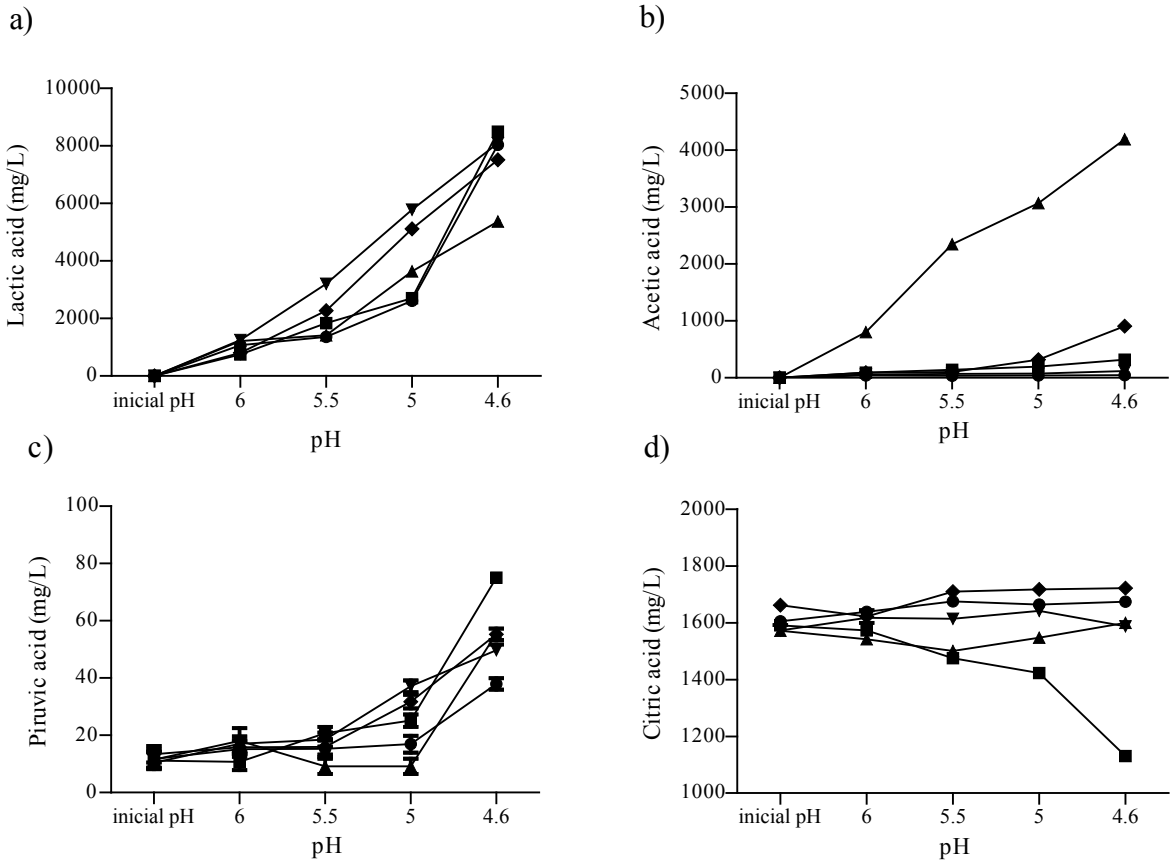

Figure 1. Lactic acid (a), acetic acid (b), pyruvic acid (c), and citric acid (d) content (mg/L) during milk fermentation by different combinations of lactic cultures at 42 °C. St treatment, fermented by *Streptocccus thermophilus* (●); La treatment, fermented by *Lactobacillus acidophilus* (■); Bb treatment, fermented by *Bifidobacterium animalis* subsp. *lactis* (▲); StLa treatment, fermented by *S. thermophilus* and *L. acidophilus* (▼); and StBb treatment, fermented by *S. thermophilus* and *B. animalis* subsp. *lactis* (♦). n = 3.

The concentration of pyruvate increased during fermentation (Figure 1c), and at the end of the incubation, it ranged from 37 to 75 mg/L. *L. acidophilus* produced the largest amount of this acid, and the peak in production occurred at the end of the fermentation process. The initial concentration of citric acid in the milk was approximately 1600 mg/L (Figure 1d), close to the amount that is found in milk (1800 mg/L) (HELLAND; WICKLUND; NARVHUS, 2004). Only *L. acidophilus* in pure culture was able to metabolize citrate at significant levels (260 mg/L). Acetic acid may be produced by lactobacilli species through the degradation of citric acid (ØSTLIE; HELLAND; NARVHUS, 2003). Ostlie, Treimo and Narvhus (2005) reported that *Lactobacillus* species produced acetic acid at concentrations of 173-1171 mg/L after

incubation at 30, 37 and 45 °C. In the present study, *L. acidophilus* produced 321.79 mg/L of acetic acid at the end of fermentation, which is close to the amount of citric acid that was degraded. *B. animalis* subsp. *lactis* was not able to metabolize citric acid when the fermentation process was carried out at 30 or 37 °C; however, all the citrate was metabolized within 24 to 48 hours of incubation at 45 °C (ØSTLIE; TREIMO; NARVHUS, 2005). In the present study, this microorganism did not metabolize citrate, and this result was likely due to the reduced incubation time (10.58 h).

3.1.2. Kinetic parameters of acidification

The acidification profile of milk fermented by *L. acidophilus* and *B. animalis* subsp. *lactis* as pure cultures or in co-cultures with *S. thermophilus* was characterized by the parameters V_{max}, t_{Vmax}, pH_{Vmax}, $t_{pH5.0,}$ and $t_{pH4.6}$ (Table 1). The highest V_{max} values were obtained in the case of the St treatment (22.18 x 10^{-3} upH/min), as well as in the cases of the StBb and StLa treatments (the values resulting from these two treatments were 21.13 x 10^{-3} upH/min and 20.39 x 10^{-3} upH/min, respectively). The lowest V_{max} value was observed in the case of the La treatment (5.28 x 10^{-3} upH/min), a result which indicates the low capacity of acidification of the *L. acidophilus* La-5 strain. The combination of probiotic strains with *S. thermophilus* significantly increased the average rate of acidification ($P \leq 0.05$) by approximately 32.23% in the case of *B. animalis* subsp. *lactis*, and by approximately 74.10% in the case of *L. acidophilus*.

L. acidophilus is a species that grows slowly in milk due to its low proteolytic activity. It is also nutritionally demanding, which explains the low V_{max} value observed. This species requires low oxygen tension, fermentable carbohydrates, proteins and amino acids, B vitamins, calcium pantothenate, folic acid, niacin and riboflavin, minerals such as magnesium, manganese and iron, and free fatty acids (SACCARO et al., 2009).

Champagne et al. (2000) and Fonseca, Beal and Corrieu (2000) studied the kinetics of acidification of different strains of *S. thermophilus* and *Lactobacillus* sp. and reported that the V_{max}, t_{Vmax}, pH_{Vmax} and $t_{pH4.5}$ values depend on the strains used during fermentation. In the present study, the t_{Vmax} value of the probiotics as pure cultures was lower in the case of Bb (8.27 h) and higher in the case of La (11.0 h). In co-culture with *S. thermophilus*, this value was 4.2 times higher in the case of *L. acidophilus* and 3.27 higher in the case of *B. animalis* subsp. *lactis*. Therefore, there was a correlation

between V_{max} and t_{Vmax}: the higher the Vmax, the lower the t_{Vmax} that was obtained. On the other hand, Mishra and Mishra (2012) examined the kinetics of acidification of *L. acidophilus*, *L. rhamnosus*, and *L. plantarum* in pure culture, in co-cultures or in mixed cultures, and reported that there was no direct relationship between V_{max} and t_{Vmax} when those types of cultures were evaluated.

Table 1. Kinetic parameters of acidification during fermentation at 42 °C.

Treatment	V_{max} (10^{-3} upH/min)	t_{vmax} (h)	pH V_{max}	$t_{pH5.0}$ (h)	$t_{pH4.6}$ (h)
St	22.18±0.08[a]	2.69±0.21[c]	5.58±0.06[a]	3.44±0.14[c]	5.91±0.17[c]
Bb	14.32±1.02[b]	8.27±0.58[b]	5.37±0.06[a]	8.82±0.49[b]	10.58±0.17[b]
La	5.28±0.08[c]	11.0±0.28[a]	5.07±0.33[a]	12.57±0.62[a]	16.07±1.32[a]
StBb	21.13±0.93[a]	2.53±0.23[c]	5.48±0.05[a]	2.49±0.21[c]	4.93±0.27[c]
StLa	20.39±0.56[a]	2.62±0.28[c]	5.59±0.09[a]	3.38±0.27[c]	5.53±0.42[c]

[a, b, c] Different letters in the same column denote significant difference ($P \leq 0.05$) among treatments. V_{max} = maximum rate of acidification; t_{max} = time required to reach Vmax; pH_{Vmax} = pH in V_{max}; $t_{pH5.0}$ = time required to reach pH 5.0; $t_{pH4.6}$ = time required to reach pH 4.6 (end of fermentation). St: milk fermented by *S. thermophilus;* Bb: milk fermented by *B. animalis* subsp. *lactis;* La: milk fermented by *L. acidophilus;* StBb: milk fermented by *S. thermophilus* and *B. animalis* subsp. *lactis;* StLa: milk fermented by *S. thermophilus* and *L. acidophilus.* n = 3.

The pH_{Vmax} value represents the concentration of H^+ ions at the moment V_{max} is reached, as well as the moment at which the maximum acid production by fermentative cultures occurs. There was no significant difference ($P \leq 0.05$) in pH_{Vmax} values for all cultures and combinations evaluated in this study.

The time required to reach the final pH (pH 4.6) varied between 4.93 and 16.07 h and was significantly ($P \leq 0.05$) different between probiotics pure cultures and co-cultures of probiotics. The lowest fermentation time was obtained by co-cultures of *S. thermophilus* with *B. animalis* subsp. *lactis*, and among the pure cultures, *S. thermophilus* was also the fastest to reach the end of fermentation (pH 4.6). The positive effect of *S. thermophilus* in the kinetics of acidification has been reported previously in other studies using probiotic strains (OLIVEIRA et al., 2009).

3.2. Evaluation during refrigerated storage

3.2.1. pH and syneresis

The pH values were affected by storage time and the type of culture used ($P < 0.05$), except for on the first and twenty-first days of cold storage (Table 2). A decrease in the pH levels for all treatments was observed over time. This behavior indicates that post-acidification occurred in the products. Some studies have shown that the pH of fermented milk during refrigerated storage may reflect changes to a greater or smaller degree, depending on the initial pH level, storage temperature and time and the post-acidifying activity of the cultures (DONKOR et al., 2006).

Table 2. pH and syneresis (%) of fermented milk during 28-day storage.

Treatment	Storage day		
	1	14	28
pH			
St	4.60±0.01 ^{Aa}	4.47±0.04 ^{Ba}	3.98±0.00 ^{Cc}
La	4.62±0.01 ^{Aa}	4.47±0.01 ^{Ba}	4.23±0.11 ^{Cab}
Bb	4.60±0.01 ^{Aa}	4.34±0.03 ^{Bb}	4.23±0.04 ^{Cab}
StLa	4.58±0.01 ^{Aa}	4.35±0.01 ^{Bcb}	4.32±0.02 ^{Ca}
StBb	4.57±0.04 ^{Aa}	4.29±0.06 ^{Bb}	4.18±0.03 ^{Cb}
Syneresis			
St	23.75±2.47 ^{Aa}	17.00±0.71 ^{Ba}	9.34±0.47 ^{Cc}
La	18.75±1.77 ^{Aa}	15.25±1.77 ^{Aa}	13.25±1.77 ^{Aabc}
Bb	22.50±2.12 ^{Aa}	18.00±0.71 ^{ABa}	15.25±1.06 ^{Bab}
StLa	23.67±0.47 ^{Aa}	18.00±1.88 ^{Ba}	17.00±0.71 ^{Ba}
StBb	22.34±3.77 ^{Aa}	19.67±2.35 ^{ABa}	11.17±1.18 ^{Bbc}

$^{a, b, c}$ Different letters in the same column denote significant difference ($P \leq 0.05$) among treatments. $^{A, B, C}$ Different letters in the same row denote significant difference ($P \leq 0.05$) among days of storage for the same treatment. St: milk fermented by *S. thermophilus;* Bb: milk fermented by *B. animalis* subsp. *lactis;* La: milk fermented by *L. acidophilus;* StBb: milk fermented by *S. thermophilus* and *B. animalis* subsp. *lactis;* StLa: milk fermented by *S. thermophilus* and *L. acidophilus.* n = 3.

There was a significant reduction in syneresis during the 28 days of refrigerated storage, with the exception of the La treatment, which kept syneresis values statistically equal ($P \leq 0.05$) during the period (Table 2). There were differences in syneresis values among treatments only on the 28th day, and the treatments with the lowest and highest syneresis values were St and StLa, respectively. These results may be related to the pH values obtained for both treatments. While the St treatment had the lowest pH value, the StLa treatment showed the highest value. These results showed that the extent of syneresis usually decreased with decreases in pH, which is in agreement with studies by Olson and Aryana (2008) and Wang et al. (2010). Despite of lower syneresis in lower pH values, this behaviour was not expected, since the pH reduction causes rearrangement of the network formed by protein in the clot. This change leads to an increase in the number of particle-particle junctions and causes the clot to retract, expelling its interstitial fluid (HASSAN et al., 1996; LUCEY, 2004; DAMIN et al., 2009).

The decrease in syneresis during the storage of the fermented milk may be due to a higher expulsion of whey in the matrix formed on the first days of storage and subsequent less syneresis on the end of storage. La Torre; Tamime and Muir (2003) also reported a decrease in syneresis in probiotic fermented milk during storage for 20 days at 5 °C. However, other authors detected increased syneresis during storage of yogurts and fermented milk (ACHANTA; ARYANA; BOENEKE, 2007; ARYANA; MCGREW, 2007).

The production of fermented milk with ideal consistency and stability for syneresis is a primary concern for the dairy industry. Syneresis is a highly undesirable phenomenon and factors affecting syneresis include total solids content, milk composition (protein, salts), the homogenization process, the type of culture, acidity resulting from the growth of bacterial cultures, and any pre-heat treatments of milk.

3.2.2. Microorganisms viability

S. thermophilus, *L. acidophilus* and *B. animalis* in pure cultures or in co-cultures showed high viability over 28 days of refrigerated storage. The *S. thermophilus* strain remained stable throughout the period and was not affected by the type of culture or by storage time ($P \leq 0.05$). This microorganism had the highest populations for 28 days, with values above 9 log CFU/mL throughout the examination period (Figure 2).

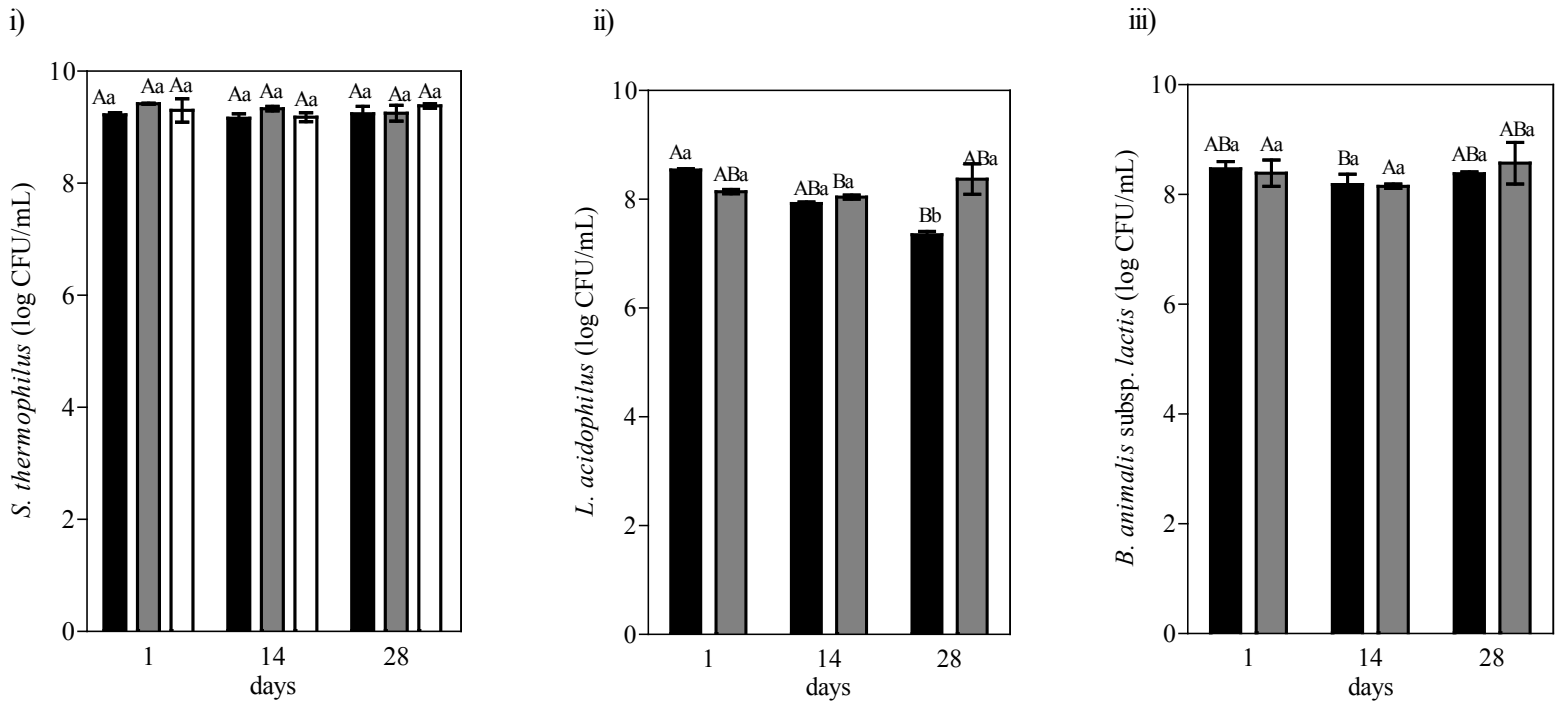

Figure 2. Population of *S. thermophilus* (i), *L. acidophilus* (ii) and *B. animalis* subsp. *lactis* (iii) (log CFU/mL) in fermented milk during refrigerated storage. i) St (■), StLa (▭) and StBb (□), ii) La (■), StLa (▭), iii) Bb (■), StBb (▭). [a, b, c] Different letters for the same storage day denote significant differences ($P \leq 0.05$) among treatments. [A, B, C] Different letters for the same treatment during refrigerated storage denote significant differences ($P \leq 0.05$) during storage. n = 3.

The *L. acidophilus* strain decreased by about one log cycle during storage when present in pure culture, with a population of 7 log CFU/mL after 28 days. On the other hand, when *L. acidophilus* was co-cultured with *S. thermophilus*, there was a significant increase ($P \leq 0.0.5$) in the population between 14 and 21 days, with a population above 8 log CFU/mL by the end of storage (Figure 2). However, the inhibition of *L. acidophilus* when the bacteria was grown in association with other bacteria (*S. thermophilus, L. delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* sp., *Lactobacillus* sp. and *Bifidobacterium* sp.) has been reported (VINDEROLA; MOCCHIUTTI; REINHEIMER, 2002; SACCARO et al., 2009). Samples of treatment containing *B. animalis* subsp. *lactis* had populations above 8 log CFU/mL throughout the storage period and, unlike that which occurred with *L. acidophilus*, its population was not affected by the presence of *S. thermophilus* (Figure 2). The minimum therapeutic effective dose, which has been suggested to be between 6–9 log CFU in the probiotic product (VASILJEVIC; SHAH, 2008), was reached during all treatments by day 28.

3.2.3. Probiotic resistance to simulated gastrointestinal conditions during refrigerated storage of fermented milk

There was a significant reduction in the population of *B. animalis* subsp. *lactis* and *L. acidophilus* over storage time for all the treatments, because the cells were more stressed and damaged by the cold storage at the end of the storage period compared to when they were newly produced (Table 3). To exert its beneficial effects in the host, the probiotic bacteria must be alive in the product at the time of consumption and must also be able to reach the large intestine in amounts high enough to facilitate colonization and proliferation at the site (SHAH, 2000).

The population of *L. acidophilus* decreased by at least 4 log cycles after 30 minutes of exposure to gastric conditions. There was a decrease in the survival of *L. acidophilus* over storage time for the both treatments (St and StLa). For the La treatment , there was no detection of viable cells on the first and fourteenth days of storage 240 minutes after beginning the assay, indicating that the presence of bile inhibited the development of microorganisms, while on the twenty-eighth day, no growth was observed after 120 minutes of exposure to gastric conditions (Table 3).

There was no recovery of viable *L. acidophilus* cells after the pH levels were increased (in the enteric phase of the assay). However, other studies showed that

recovery of this microorganism occurred after pH elevation (THANTSHA et al., 2009; BURITI; CASTRO; SAAD, 2010), and that it attributed to the temporary stress of probiotic cells due to the low pH levels. In these studies, the probiotic was in the presence of prebiotics (inulin, whey protein concentrate), or it was microencapsulated, which aided its survival at the low pH. In the present study, there were no compounds or ingredients to protect the probiotic cells and avoid cell death in the presence of low pH levels. Thus, to exert a therapeutic effect and to ensure probiotic survival during gastrointestinal transit, this strain must be protected.

Madureira et al. (2005) found no change in the survivability of *L. acidophilus* Lac-1 and *L. acidophilus* Ki in the case of whey cheese. These strains maintained viability levels greater than 7 log CFU/mL and 8 log CFU/mL, respectively, when exposed to pepsin at pH 2.5-3.0 for 60 to 120 minutes. In this study, however, the pH used in the gastric phase (1.4-1.9), probably contributed to a relatively low survival of *L. acidophilus* compared to that observed by other authors using pH levels of 2 and above. Buriti, Castro and Saad (2010) used pH values between 1.4 and 1.9 and also observed a reduction in the population of *L. acidophilus* La-5 after 120 minutes of exposure to gastric juice, especially in the mousse without the addition of prebiotics.

Despite the general definition that probiotics are live microorganisms, a variety of biological responses have been reported from administering dead, frequently heat-killed, probiotics to various mammalian and avian species. Live probiotic cells might well influence the gastrointestinal microbiota and have an immunomodulating effect, whereas the components of dead cells could exert an anti-inflammatory response. However, the relative importance of these two effects is difficult to assess since an immunomodulating response of both live and dead probiotic cells has been extensively investigated. Dead probiotic cells are not a necessary requirement to generate a biological response but they may be sufficient (ADAMS et al., 2010).

The population of *B. animalis* subsp. *lactis* in the treatment fermented by the pure culture (Bb) had no significant ($P>0.05$) decrease on the first day of storage after exposure to gastric and enteric juices, but after 14 days, the viability significantly decreased ($P\leq0.05$) – by almost 1 log cycle – and on the last day of storage, the population was 2 log cycles smaller at the end of the assay (Table 3). In the case of the StBb treatment, the bifidobacteria population decreased ($P\leq0.05$) by almost 1 log cycle on the first day of storage and decreased ($P\leq0.05$) by at least 4 log cycles on the

fourteenth and twenty-eighth days of storage after exposure to simulated gastrointestinal conditions.

Table 3. Survival of *L. acidophilus* and *B. animalis* subsp. *lactis* BB-12 (log CFU/mL) in milk fermented by pure culture (La and Bb) or by co-culture with *S. thermophilus* (StLa and StBb) during exposure to *in vitro* gastric conditions for 30 and 120 min (pH 1.4-1.9), and enteric conditions for 240 min (pH 4.3-5.2) and 360 min (pH 6.7-7.5), after 1 (D1), 14 (D14) and 28 (D28) days of refrigerated storage.

		t0	t30	t120	t240	t360
La	D1	8.42 ± 0.02^{Aa}	4.60 ± 0.54^{Ba}	3.96 ± 0.86^{Ba}	$<1^{Ca}$	$<1^{Ca}$
	D14	7.96 ± 0.09^{Aa}	2.91 ± 0.04^{Bb}	2.81 ± 0.71^{Bb}	$<1^{Ca}$	$<1^{Ca}$
	D28	7.39 ± 0.06^{Aa}	3.63 ± 0.01^{Bab}	$<1^{Cc}$	$<1^{Ca}$	$<1^{Ca}$
StLa	D1	8.33 ± 0.03^{Aa}	4.94 ± 0.81^{Ba}	3.87 ± 0.03^{Ca}	3.52 ± 0.02^{Ca}	2.46 ± 0.07^{Da}
	D14	8.28 ± 0.05^{Aa}	5.54 ± 0.81^{Ba}	3.04 ± 0.02^{Db}	$<1^{Db}$	$<1^{Db}$
	D28	8.32 ± 0.01^{Aa}	3.74 ± 0.16^{Bb}	$<1^{Cb}$	$<1^{Cb}$	$<1^{Cb}$
Bb	D1	8.37 ± 0.05^{Aa}	8.04 ± 0.06^{Aa}	8.14 ± 0.15^{Aa}	8.04 ± 0.04^{Aa}	8.12 ± 0.12^{Aa}
	D14	8.52 ± 0.12^{Aa}	7.72 ± 0.21^{Ab}	8.18 ± 0.26^{Aa}	7.68 ± 0.17^{ABb}	7.72 ± 0.17^{Ab}
	D28	8.18 ± 0.07^{Aa}	8.03 ± 0.27^{Aa}	7.77 ± 0.22^{Ab}	6.68 ± 0.23^{Bb}	6.26 ± 0.38^{Bc}
StBb	D1	8.17 ± 0.07^{Aa}	8.09 ± 0.02^{Aa}	7.92 ± 0.01^{Aa}	7.58 ± 0.03^{Aa}	7.36 ± 0.41^{Aa}
	D14	8.01 ± 0.56^{Aa}	6.70 ± 0.17^{Bb}	6.65 ± 0.03^{Bb}	5.62 ± 0.04^{Cb}	5.39 ± 0.06^{Cb}
	D28	8.31 ± 0.09^{Aa}	6.68 ± 0.08^{Bb}	6.73 ± 0.03^{Bb}	5.04 ± 0.04^{Cc}	4.99 ± 0.06^{Cc}

[a, b, c] Different lower case letters in the same column denote significant differences ($P\leq0.05$) among days of storage for the same treatment. [A, B, C] Different capital letters in the same row denote significant differences ($P\leq0.05$) among sampling periods of the *in vitro* assay. n = 3.

B. animalis subsp. *lactis* was more resistant to the acidic condition used in the assay compared to *L. acidophilus*. Bedani, Rossi and Saad (2013) reported that *B. animalis* BB-12 was more resistant than *L. acidophilus* La-5 to *in vitro* simulation of the gastrointestinal tract in samples of a fermented soy product. Madureira et al. (2005) analyzed the survival of probiotics and reported that few bifidobacteria strains were less resistant to acid than the lactobacilli. On the other hand, Guo et al. (2009) found a higher sensitivity of bifidobacteria to acid compared to lactobacilli. *B. animalis* strains are clearly more acid resistant than other strains of *Bifidobacterium* (MÄTTÖ et al.,

2006). Therefore, it seems that probiotic resistance under simulated gastrointestinal tract conditions depends on the strain that is being evaluated.

3.2.4. Sensory analysis

Sensory evaluation of fermented milk after processing showed significant variation ($P \leq 0.05$) among the treatments for all attributes in both storage times. The type and composition of the culture affected the acceptability of fermented milk samples. The results ranged from 3 (dislike moderately) to 7 (like moderately). The La treatment was the one most rejected (Table 4).

Fermented milk is characterized as a viscous and smooth gel with a slight and refreshing sour taste, and the fermented milk should therefore be evaluated in terms of appearance, flavor, texture and overall appearance (HEKMAT; REID, 2006). The overall appearance and consistency attributes were statistically similar ($P > 0.05$) among the treatments after both storage periods with the exception of the La treatment, which received the lowest scores at 14 days of storage. Good quality yogurt should maintain strong curd integrity without any sign of shrinkage, disintegration into lumps, or whey-off (SRISUVOR et al., 2013). The granule formation in the La-fermented milk may have caused the lower scores given for the appearance attribute. The formation of granules is one of the technical defects of fermented milk and may be caused by various factors, such as the use of high temperature incubation, an excess in the whey protein content, and the type of starter culture used. The low scores given for the consistency attributed to the La treatment (Table 3) can be ascribed to longer fermentation time (16.07 h) obtained during this treatment compared to the other treatments (5.91, 10.58, 4.93 and 5, 53 h, respectively, for the St, Bb, and StLa StBb treatments). In the manufacturing of fermented milk, slow acidification leads to the formation of a weak gel which is possibly related to the rate and extent of solubilization of colloidal phosphate calcium before the formation of the protein network (LUCEY, 2004).

In the sensory evaluation, samples of the La treatment also received the lowest scores for the odor attribute, and it differed significantly ($P \leq 0.05$) from the others. Milk fermented by *L. acidophilus* is characterized by poor flavor, since this microorganism synthesizes an alcohol dehydrogenase that converts acetaldehyde into ethanol (MARSHALL; COLE, 1983). The major differences among the samples of fermented milk were observed in the case of acceptability and flavor attributes. Flavor is one of the

most important properties of food acceptability and consumer preference (CHENG, 2010). When it came to the flavor attribute, samples from La and Bb treatments received the lowest and highest scores, respectively, after 14 days of storage. After 28 days, the score of the La treatment remained the lowest; it differed significantly ($P \leq 0.05$) from the other treatments.

Table 4. Scores (1-9 points) of sensory evaluation of fermented milk at 14 and 28 days after manufacturing.

Attribute	Day	Treatment				
		St	Bb	La	StBb	StLa
Overall appearance	14	7.22±1.39 ^{Aa}	7.31±1.25 ^{Aa}	5.24±1.77 ^{Bb}	7.24±1.44 ^{Aa}	6.97±1.32 ^{Aa}
	28	7.34±1.20 ^{Aa}	7.44±1.18 ^{Aa}	6.22±1.65 ^{Ba}	7.47±1.25 ^{Aa}	7.24±1.21 ^{Aa}
Consistency	14	7.01±1.64 ^{Aa}	7.17±1.47 ^{Aa}	5.28±1.83 ^{Ba}	7.31±1.37 ^{Aa}	7.07±1.44 ^{Aa}
	28	7.27±1.27 ^{Aa}	7.19±1.22 ^{Aa}	5.97±1.67 ^{Ba}	7.41±1.19 ^{Aa}	7.17±1.34 ^{Aa}
Odor	14	6.84±1.40 ^{Aa}	6.78±1.27 ^{Aa}	5.67±1.63 ^{Ba}	6.53±1.70 ^{Aa}	6.48±1.38 ^{ABa}
	28	6.88±1.43 ^{ABa}	6.71±1.42 ^{ABa}	6.05±1.56 ^{Ba}	7.05±1.48 ^{Aa}	6.74±1.40 ^{ABa}
Flavor	14	5.20±1.97 ^{ABa}	5.93±2.06 ^{Aa}	3.41±2.01 ^{Ca}	5.51±1.90 ^{ABa}	5.20±1.97 ^{ABa}
	28	5.63±1.83 ^{Aa}	6.20±1.79 ^{Aa}	3.98±1.95 ^{Ba}	6.19±1.80 ^{Aa}	5.37±1.85 ^{Aa}
Overall acceptability	14	5.81±1.73 ^{ABa}	6.26±1.80 ^{Aa}	3.83±1.87 ^{Cb}	6.12±1.66 ^{Ab}	5.66±1.77 ^{Ba}
	28	6.22±1.55 ^{Aa}	6.51±1.62 ^{Aa}	4.58±1.90 ^{Ba}	6.71±1.51 ^{Aa}	5.90±1.71 ^{Aa}

$^{a, b}$ Different letters in the same column for a same treatment denote significant difference ($P \leq 0.05$) between days of storage. $^{A, B, C}$ Different letters in the same row denote significant difference ($P \leq 0.05$) among treatments for the same parameter. St: milk fermented by *S. thermophilus;* Bb: milk fermented by *B. animalis* subsp. *lactis;* La: milk fermented by *L. acidophilus;* StBb: milk fermented by *S. thermophilus* and *B. animalis* subsp. *lactis;* StLa: milk fermented by *S. thermophilus* and *L. acidophilus.* n = 60.

The overall acceptability of the samples varied among the fermented milk products ($P \leq 0.05$) over time and among treatments. Again, La treatment samples had the lowest overall acceptability scores after 14 and 28 days of storage. After 28 days of storage, only the score of La treatment was significantly different ($P \leq 0.05$) from the others. Surprisingly, fermented milk prepared with *B. animalis* (StBb and Bb treatments) had the highest scores for acceptability after both storage times. This behavior was not expected, since bifidobacteria produce acetic and lactic acid at a ratio of 3:2 during fermentation and storage of products, which normally leads to the rejection of the product by the consumer. However, the fermentation temperature used resulted in a lower amount of acetic acid and a higher amount of lactic acid produced.

This change may have contributed to the higher product acceptability scores given by consumers. The low scores received by the fermented milks samples can be justified because they are not commercial product and there was no addition of sugar and stabilizers during their manufacturing. Nervertheless, the sensory analysis gave important information about the impact of culture combination over sensory charactristics of fermented milk.

3.2.5. Organic acid production

There were significant differences ($P \leq 0.05$) for the concentration of lactic, acetic, citric and pyruvic acids among treatments on both period of storage (Table 5).

During storage, the production of lactic acid was observed; however, the concentration of acetic, citric and pyruvic acids remained relatively constant. The highest levels of lactic acid were detected when in the La treatment, both at 14 (11.154 mg/L) and at 28 (12.237 mg/L) days of analysis. The higher concentration of acetic acid was obtained for the Bb treatment on both days of storage (5.713 and 5.901 mg/L, after 14 and 28 days, respectively).

The profile of organic acids during storage was correlated with the sensory evaluation of fermented milk. The major differences between the samples of fermented milk were observed for overall acceptability and flavor attributes. The lowest scores for flavor and acceptability received by the La treatment can be related to the higher quantities of lactic acid and lower amount of citric acid in this sample. There was a high negative correlation between the amount of lactic acid and the scores received for flavor (r = -0.911, $P \leq 0.05$and r = -0.904, $P \leq 0.05$ after 14 and 28 days of storage, respectively) and for overall acceptability (r = -0.879, $P \leq 0.05$ and r = -0.890, $P \leq 0.05$ after 14 and 28 days of storage, respectively) in the sensory evaluation of fermented milk. Moreover, there was a strong positive correlation between the amount of citric acid and flavor scores (r = 0.971 and r = 0.959 after 14 and 28 days of storage, respectively) and overall acceptability (r = 0.989 and r = 0.974 after 14 and 28 days of storage, respectively) in the sensory evaluation of fermented milk. The correlation test indicated a poor correlation (r<0.4) between the quantities of the other two acids (acetic and pyruvic acid), and the attributes for flavor and overall acceptability.

Table 5. Concentration (mg/L) of organic acids in fermented milk samples during refrigerated storage at 42 °C.

		Lactic	Acetic	Citric	Pyruvic
D14	St	10629 ± 23.40 [b]	49 ± 2.76 [e]	1764 ± 33.19 [a]	48 ± 0.73 [d]
	La	11154. ± 12.46 [a]	564 ± 0.58 [c]	835 ± 2.82 [c]	136 ± 1.96 [a]
	Bb	9849 ± 37.15 [d]	5713 ± 2.38 [a]	1802 ± 12.42 [a]	83 ± 2.01 [b]
	StLa	10643 ±35.78 [b]	165 ± 1.92 [d]	1592 ± 1.25 [b]	72 ± 2.27 [c]
	StBb	10161 ± 36.89 [c]	1058 ± 4.31 [b]	1752 ± 10.14 [a]	65 ± 1.84 [c]
D28	St	11226 ± 25.86 [c]	48 ± 1.78 [e]	1750 ± 14.03 [a]	35 ± 1.83 [e]
	La	12236 ± 28.78 [a]	459 ± 5.96 [c]	1000 ± 15.21 [c]	138 ± 0.21 [a]
	Bb	10605 ± 2.70 [d]	5901 ± 2.22 [a]	1729 ± 5.56 [a]	86 ± 2.38 [b]
	StLa	11954 ±29.42 [b]	185 ± 1.20 [d]	1588 ± 9.55 [b]	61 ± 1.61 [d]
	StBb	10532 ± 50.39 [d]	1079 ± 6.12 [b]	1755 ± 13.22 [a]	73 ± 1.75 [c]

[a, b, c] Different letters in the same column denote significant difference ($P \leq 0.05$) among treatments for the same day of storage. St: milk fermented by *S. thermophilus;* Bb: milk fermented by *B. animalis* subsp. *lactis;* La: milk fermented by *L. acidophilus;* StBb: milk fermented by *S. thermophilus* and *B. animalis* subsp. *lactis;* StLa: milk fermented by *S. thermophilus* and *L. acidophilus.* n = 3. D14: 14 days of storage, D28: 28 days of storage.

Milk fermented by strains belonging to the genus *Bifidobacterium* may have undesirable taste due to the presence of acetic acid, which is formed as a result of the metabolism of this microorganism (DONKOR et al., 2007). In this study, however, the amount of acetate that was produced was not large enough to adversely affect the flavor of the products. Moreover, *B. animalis* can produce some amount of acetoin instead of acetic acid to maintain homeostasis of the medium (TSAU; GUFFANTI; MONTVILLE, 1992).

4. CONCLUSIONS

The kinetic acidification parameters of fermented milk was influenced by the composition of starter culture, and the best kinetic parameters (short time to reach the final pH, high V_{max} and low t_{vmax}) were obtained for the samples fermented by *S. thermophilus* in pure or co-culture. The composition of the starter culture also affected the characteristics of the products during the refrigerated storage in the following ways:

• there was an increase in syneresis over time, with the St treatment sample being the most stable;

• the population of microorganisms remained stable during storage, except in the case of the La treatment;

• *in vitro* resistance of probiotics decreased over storage time, and the resistance of bifidobacteria was superior than that of *L. acidophilus*;

• samples fermented by *B. animalis* subsp. *lactis* and *L. acidophilus* had the highest and lowest sensory attribute scores, respectively, and the scores were correlated to the amounts of lactic and citric acids.

ACKNOWLEDGMENTS

This work was in part supported by FAPESP (São Paulo Research Foundation, grant 2009/12808-6).

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Capítulo 3

Este trabalho será submetido ao *British Food Journal*

INCORPORATION OF FRUIT FLOURS INTO FERMENTED MILK: ACIDIFICATION PROFILE, VIABILITY OF PROBIOTICS AND GASTROINTESTINAL TOLERANCE

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ABSTRACT

The effect of the addition of apple, banana and grape flours (1%) on the acidification profile, bacterial counts, total titratable acidity, post-acidification and resistance of probiotics to simulated gastric and enteric conditions in milk fermented by ABT-4 culture (*Streptococcus thermophilus, Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus acidophilus* La-5) was evaluated during a 28-day storage period. Milk supplementation with fruit flour affected only the acidification rate (V_{max}) and time to reach V_{max} (t_{vmax}), and there was no effect on fermentation time. There was a decrease in the pH level and an increase in titratable acidity, regardless of the addition of flours. The population of probiotics was not affected by the milk supplementation with fruit flours during 28 days of storage. Decreased probiotic survival during *in vitro* gastrointestinal simulation was observed in all the treatments. Bb-12 resistance to the artificial gastrointestinal juices was higher than for La-5, since the Bb-12 and La-5 populations decreased approximately 1 log CFU/mL and 4 log cfu/mL, respectively, throughout storage period. Nonetheless, the presence of fruit flours enhanced *L. acidophilus* survival, while the resistance of *B. animalis* subsp. *lactis* during the *in vitro* assay was not affected by the addition of fruit flours.

Key words: viability, probiotics, kinetics parameters of acidification, gastrointestinal tract, dairy products.

1. INTRODUCTION

Fermented milk is one of the most popular dairy products, and their sales have been increasing due to the diversification in the range of fermented milk-like products, including reduced fat content products, probiotics, drinkable fermented milk, etc (STAFFOLO et al., 2004). The addition of natural products to fermented milk, such as fruits, fibers and flours, can enhance milk's nutritional properties and also improve probiotic survival during storage (SENDRA et al., 2008; ESPIRITO-SANTO et al., 2012; ZARE et al., 2013). Probiotics are defined as live microorganisms that are able to colonize the gastrointestinal tract, and, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). The strains that are most widely used in fermented dairy products belong either to the genus *Bifidobacterium* or to the *Lactobacillus casei* and *Lactobacillus acidophilus* groups (VINDEROLA et al., 2011).

It is widely accepted that, in order to achieve their claimed beneficial effects, probiotics must be present at a minimum level (10^6-10^7 CFU/mL) in the functional fermented milk (BURITI; CASTRO; SAAD, 2010). The current Brazilian legislation states that the minimum viable quantity of probiotic culture should be between 10^8 and 10^9 CFU per daily serving (ANVISA, 2013). However, a number of factors can affect probiotic cell viability in fermented milk products, including the chemical ingredients used for product manufacture (VINDEROLA et al., 2002), the presence of fruit pulps (KAILASAPATHY; HARMSTORF; PHILLIPS, 2008), the possible interactions between starter cultures and probiotic adjuncts (MISHRA; MISHRA, 2012), the addition of yeast (CASAROTTI et al., 2013) and prebiotics (MADHU; AMRUTHA; PRAPULLA, 2012). Moreover, it is more appropriate to evaluate the survival of probiotics in unfavorable conditions that simulate the environment found in the gastrointestinal tract (GIT), than to evaluate their survival in the food only during its shelf life (RANADHEERA et al., 2012).

Orally delivered probiotics must be able to withstand the different challenges found along the GIT – particularly acidic pH levels and gastric enzymes in the stomach, as well as bile, pancreatin and other intestinal enzymes in the small intestine throughout the entire food storage period in order to persist, at least partially, within the host's body (FONTANA et al., 2013). Different strategies have been employed to obtain higher probiotic survival rates in the GIT, such as microencapsulation (GEBARA et al., 2013),

milk supplementation with prebiotics, co-culture with other probiotics, and/or the use of a more appropriate food matrix (RANADHEERA et al., 2012). Tests of survival under simulated gastric and enteric conditions is among the *in vitro* assays that are most commonly suggested for the evaluation of the strain's probiotic potential (GBASSI et al., 2011).

However, it is more feasible to study the tolerance to gastrointestinal conditions with strains that have been incorporated into the final food product (SCHILLINGER; GUIGAS; HOLZAPFEL, 2005), because various factors associated with the carrier foods (including ingredients, the manufacturing process, physicochemical characteristics, and storage conditions) can affect the functional properties of probiotics. However, there has been little study on the effect of different food carriers on the gastrointestinal tolerance of probiotic bacteria (RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010; SAXELIN et al., 2010; RANADHEERA et al., 2012).

The addition of inulin was proven to increase the resistance of probiotics to artificial GIT juices (BURITI; CASTRO; SAAD, 2010). In addition, it is important to find other ingredients that can improve probiotic survival both in the product and during transit through GIT.

Therefore, incorporating fruit flours into fermented milk seems to be a useful way to obtain products with high nutritional value. To the best of our knowledge, this is the first study that demonstrates the protective effect of fruit flours on the simulated gastrointestinal resistance of *L. acidophilus*. The aims of this study were to evaluate whether fruit flours (apple, banana and grape) affect the viability of probiotics in fermented milk during different periods of refrigerated storage, and also to evaluate these effects when the probiotics are exposed to conditions that simulate the passage through the gastrointestinal tract. In addition, the kinetics of acidification were assessed, as well as the pH values and titratable acidity during the storage period of the products.

2. MATERIAL AND METHODS

2.1. Cultures and ingredients

Skimmed milk powder (SMP) (Nestlé, Araçatuba, São Paulo, Brazil), ABT-4 culture (Chr. Hansen, Valinhos, São Paulo, Brazil), composed of *Streptococcus*

thermophilus, Lactobacillus acidophilus La-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12, commercial apple, banana and grape flours (Macçã Company, Fraiburgo, Santa Catarina, Brazil), were used in this study. According to the manufacturer, apple flour contains 86% carbohydrates, 2% protein, 8% dietary fiber, 0% fat; banana flour contains 65% carbohydrates, 4.5% protein, 11% dietary fiber, 0.7% fat; and grape flours contains 67% carbohydrates, 3% protein, 7% dietary fiber, 0% fat.

2.2. Inoculum and milk base preparation

The ABT-4 pre-culture was prepared by suspending 700 mg of freeze-dried culture in 50 mL of sterilized milk (10% total solids), followed by activation at 42 °C for 30 min. Each 250 mL of milk was inoculated with 1.0 mL of the activated pre-culture. This procedure gave initial counts after milk inoculation of approximately 10^6 CFU/mL for *L. acidophilus* La-5 and 10^7 CFU/mL for *S. thermophilus* and *B. animalis* subsp. *lactis* BB-12.

Skim powder milk (Nestlé, Araçatuba, Brazil) was reconstituted at 13 g/100 g of distilled water and divided into equal portions. Three milk bases were supplemented with 1% apple (FMA), banana (FMB) or grape (FMG) flour, and the fourth milk base was not supplemented (control - FMC). All of the milk bases were thermally treated at 90 °C for 10 min in a water bath and transferred to 500-mL sterile Schott flasks inside a laminar flow chamber, and stored at 4 °C for 24 h before use.

2.3. Fermentations and kinetic parameters

Inoculated milk samples were transferred to a water bath connected to a CINAC (Cynetique d´acidificacion, Alliance Instruments, Frepillon, France) system that allows for the continuous measurement and recording of the pH levels as well as the evaluation of the acidification rate throughout the fermentation process. Batch fermentations were performed in three separate independent trials at 42 °C up to a pH of 4.6, which was selected as the condition for stopping the fermentation. From the data collected, the acidification rate (V_{max}) was calculated as the time variation of the pH (dpH/dt) and expressed as 10^{-3} pH units/min. During the incubation period, the following kinetic parameters were also calculated: (i) t_{max} (h), time in which V_{max} was reached; (ii) $t_{pH5.0}$

(h), the time required to reach pH 5.0; (iii) $t_{pH4.6}$ (h), and the time required to reach pH 4.6 (i.e., to complete the fermentation).

When the fermentation process was completed, the samples were cooled down to 15 °C in an ice bath, and the clot was then broken down using a stainless steel perforated disk with up and down movements for approximately 1 min. The product was placed in 80-mL sterile plastic cups and stored at 4 °C for 28 days.

2.4. Product characterization

Fermented milk post-acidification was determined 1 day after the fermentation was complete and after 14 and 28 days of storage at 4 °C. It was determined through a pH measurement in triplicate using a pHmeter model PG1800 (Gehaka, São Paulo, Brazil). Acidity, expressed as lactic acid per 100 grams, was determined in triplicate for each trial using a titration 0.1-N NaOH solution (AOAC, 2005).

Bacterial counts of each treatment were carried out in duplicate after 1, 14 and 28 days of storage. *S. thermophilus* colonies were enumerated in M17 agar (Himedia, Mumbai, India). *L. acidophilus* counts were carried out in MRS agar (Acumedia, Lansing, MI, USA) with a bile solution added (Sigma-Aldrich, St. Louis, USA), and *B. animalis* subsp. *lactis* counts were carried out in MRS agar (Acumedia, Michigan, USA) with 0.2% lithium chloride and 0.3% sodium propionate (Sigma-Aldrich, St. Louis, USA) added. The M17, MRS-bile and MRS-LP media were prepared according to IDF (1997), IDF (1995) and Vinderola and Reinheimer (1999), respectively. Plates of *S. thermophilus* and *L. acidophilus* were incubated at 37 °C for 48 h under aerobic conditions. Plates of *B. animalis* subsp. *lactis* were incubated under anaerobic conditions provided by Anaerobac (Probac, São Paulo, Brazil). Cell concentration was expressed as log CFU/mL of fermented milk.

The evaluation of probiotic survival in fermented milk samples subjected to gastric and enteric simulated conditions was performed in duplicate after 1, 14 and 28 days of refrigerated storage according to the method described by Buriti, Castro and Saad (2010), with modifications. Fermented milk was diluted in a 0.5% NaCl solution, and 10 mL of this solution was transferred to 3 sterile flasks. Pepsin (3 g/L) (from porcine stomach mucosa, Sigma-Aldrich, St. Louis, USA) and lipase (0.9 mg/L) (Amano lipase F-AP15 from *Rhyzopus oryzae*, Aldrich Chemical Company, Milwaukee, USA) solutions were added to the samples, and the pH was adjusted to 2.0–

2.2 with 0.5-N HCl. Flasks were incubated at 37 °C for 2 h (gastric phase). In the next step of the assay, the pH levels of the samples were increased to 4.3–5.2 using an alkaline solution containing bile (10 g/L) (bovine bile, Sigma-Aldrich) and pancreatin (1 g/L) (pancreatin from porcine pancreas, Sigma-Aldrich). Samples were incubated again at 37 °C for 2 h (enteric phase 1). Finally, the pH levels were increased to 7.0–7.3 using the same alkaline solution. Bile and pancreatin concentrations were adjusted (10 g/L and 1 g/L, respectively), and samples were incubated once more at 37 °C for 2 h (enteric phase 2), thus achieving 6 h of assay. The enumeration of probiotics was performed in aliquots (1 mL) collected from duplicate samples after 0, 2, 4 and 6 h, using the same conditions described previously.

2.5. Statistical analyses

Two-way ANOVA was employed to analyze the results using STAT Software version 2.0. Mean values were compared using the Tukey test at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Kinetic parameters of acidification

The acidification profiles of the control and of the milk with fruit flours and fermented by the ABT-4 cultures were characterized using the parameters V_{max}, t_{vmax}, pH_{Vmax}, $t_{pH5.0}$ and $t_{pH4.6}$ (Table 1), and the acidification curves are shown in Figure 1. The V_{max} was significantly higher, and the t_{vmax} was significantly lower ($P \leq 0.05$) for fermented milk without flour incorporation, with exception for FMG regarding t_{vmax}. Results for pH V_{max}, $t_{pH5.0}$ and $t_{pH4.6}$ were statistically ($P > 0.05$) equal for all of the treatments (Table 1).

In this study, the larger amount of total solids in milk resulted by the addition of fruit flours did not enhanced the fermentation time (Figure 1). The buffering capacity of a fermented dairy product is directly proportional to its total solids content, which can result in higher kinetic parameters and longer fermentation times (VARGHESE; MISHRA, 2008). On the other hand, Espírito-Santo et al. (2012) reported a shorter fermentation time, and also reported that the maximum rate of acidification (V_{max}) was significantly reduced ($P \leq 0.05$) by the addition of passion fruit peel powder to both skim

and whole milk. This result can likely be ascribed to the presence of high buffering capacity substances in the passion fruit peel, such as organic acids and phenolic compounds. In other studies that followed the kinetics of acidification during fermentation, the addition of pea flour increased acid production by a pure culture of *L. rhamnosus* (ZARE et al., 2013), and yogurt culture was stimulated by lentil flour (ZARE et al., 2011).

Table 1. Kinetic parameters of acidification of fermented milk control and fermented milk with fruit flours.

Treatment	V_{max} (10^{-3} upH/min)	t_{vmax} (h)	pH_{Vmax}	$t_{pH5.0}$ (h)	$t_{pH4.6}$ (h)
FMC	21.70±0.88[a]	1.87±0.00[b]	5.70±0.03[a]	2.73±0.07[a]	4.39±0.10[a]
FMA	17.25±0.51[b]	2.04±0.05[a]	5.61±0.06[a]	2.92±0.10[a]	4.50±0.19[a]
FMB	18.39±0.24[b]	1.98±0.04[a]	5.70±0.15[a]	2.83±0.06[a]	4.42±0.04[a]
FMG	19.71±0.86[b]	1.97±0.04[ab]	5.69±0.11[a]	2.80±0.09[a]	4.41±0.18[a]

[a, b, c] Different letters in the same column denote significant difference ($P \leq 0.05$) among treatments. FMC (fermented milk control, without addition of flour), FMA (fermented milk with addition of 1% apple flour), FMB (fermented milk with addition of 1% banana flour) and FMG (fermented milk with addition of 1% grape flour). V_{max} = maximum rate of acidification; t_{max} = time to reach V_{max}; pH_{Vmax} = pH in V_{max}; $t_{pH5.0}$ = time required to reach pH 5.0; $t_{pH4.6}$ = time required to reach pH 4.6 (end of fermentation). n = 3.

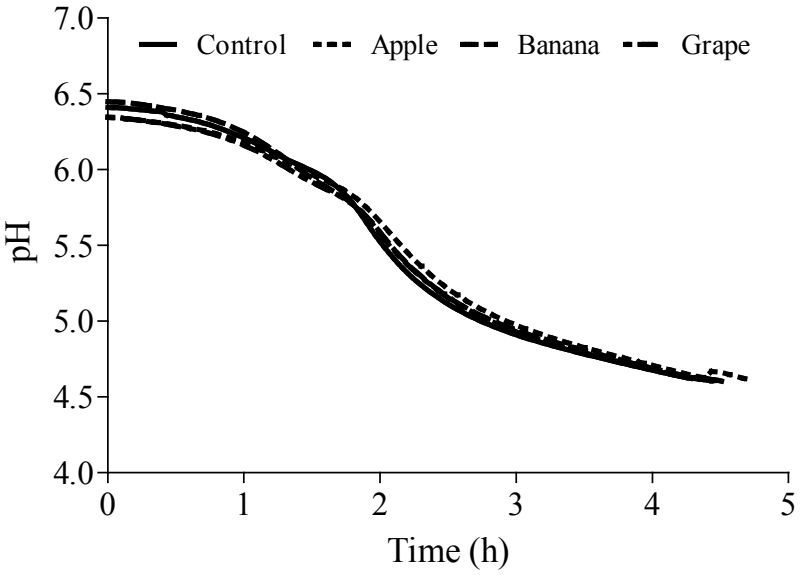

Figure 1. Acidification curves obtained during fermentation at 42 °C.

3.2. Post-acidification and titratable acidity

Acid production by microorganisms causes a decrease in pH during fermentation at 42 °C, and this decrease continues during storage at 4 °C (SHAH, 2000). As a result, there was an expected pH difference after 28 days of storage in all samples (Table 2). The pH value decreased slightly (by 0.25 to 0.32 units), while titratable acidity increased by 0.18 to 0.19% over the 28-day storage period. The decrease in pH levels was greater in the control product than in the samples supplemented with flours.

After 1 and 14 days of cold storage, the fermented milk with apple flour was found to have titratable acidity that was significantly lower ($P \leq 0.05$) than the other treatments (Table 2), whereas no statistically significant effect on pH ($P > 0.05$) could be ascribed to the treatments. The titration acidity increased at 14 days of storage and stayed almost the same after 28 days, and the only insignificant effect in this case ($P > 0.05$) was that of the fermented milk with banana flour.

At the end of 4 weeks of storage (28 days), post-acidification exhibited almost the same results as those of day 14 ($P \leq 0.05$). FMA and FMG presented pH levels that were significantly higher ($P \leq 0.05$) than those of the control. A positive and strong correlation ($r = 0.955$) was observed between the pH value and *B. animalis* subsp. *lactis* counts throughout the storage period, a result which indicates that this strain may reduce the production of organic acids in the presence of apple and grape flours. However, other studies reported that the addition of fibers (apple, wheat, bamboo, inulin, orange, lemon, date and wheat bran) did not affect the pH and acidity of yogurts (STAFFOLO et al., 2004; SENDRA et al., 2008; HASHIM; KHALIL; AFIFI, 2009).

3.3. Microorganisms viability

Inoculation rate of *S. thermophilus* and probiotic strains in milk before fermentation showed no significant difference ($P > 0.05$) among the treatments (data not shown). On day 1, viable counts of *L. acidophilus* and *B. animalis* subsp. *lactis* in all samples varied from 7.36 to 7.56 log CFU/mL and from 8.29 to 8.41 log CFU/mL, respectively, and these counts decreased over the 28-day storage period by 0.15-0.29 log CFU/mL and 0.13-0.46 CFU/mL, respectively (Table 3).

Table 2. Titratable acidity and post-acidification (pH) of control and fermented milk with fruit flour during refrigerated storage.

Treatment	Storage days		
	1	14	28
Titratable acidity			
FMC	0.83 ± 0.01^{cA}	0.98 ± 0.01^{bA}	1.01 ± 0.00^{aA}
FMA	0.80 ± 0.02^{cB}	0.94 ± 0.01^{bB}	0.98 ± 0.00^{aA}
FMB	0.82 ± 0.01^{bAB}	0.98 ± 0.01^{aA}	1.01 ± 0.02^{aA}
FMG	0.84 ± 0.02^{cA}	0.98 ± 0.00^{bA}	1.02 ± 0.03^{aA}
pH			
FMC	4.59 ± 0.04^{aA}	4.35 ± 0.03^{bA}	$4.27\pm0,03^{cC}$
FMA	4.61 ± 0.01^{aA}	4.39 ± 0.03^{bA}	$4.36\pm0,02^{bA}$
FMB	4.63 ± 0.02^{aA}	4.36 ± 0.03^{bA}	$4.30\pm0,01^{cBC}$
FMG	4.59 ± 0.01^{aA}	4.38 ± 0.03^{bA}	$4.34\pm0,03^{bB}$

Values are expressed as mean ± SD. FMC (fermented milk control, without addition of flour), FMA (fermented milk with addition of 1% apple flour), FMB (fermented milk with addition of 1% banana flour) and FMG (fermented milk with addition of 1% grape flour). $^{a, b, c}$ Different lowercase letters in a row denote significant differences within the same treatment during the storage period ($P\leq0.05$). $^{A, B, C}$ Different capital letters in a column denote significant differences among treatments ($P\leq0.05$). n = 3.

Even though certain variations in the probiotic populations were observed, these changes were of little microbiological significance, since they were below 1 log CFU/mL. The slight stimulatory effect of apple and banana flours on the probiotic strains during the storage of fermented milk may be caused by the high contents of pectins and fructooligosaccharides found in apple and banana flours (ESPIRITO-SANTO et al., 2012). This finding is agreement with those observed using lentil and pea flours and orange by-products in combination with *L. acidophilus* or bifidobacteria (SENDRA et al., 2008; AGIL et al., 2013; ZARE et al., 2013). In contrast, the lack of stimulation seen by grape flour regarding probiotic counts despite of the presence of pectin could be ascribed to the simultaneous presence of inhibitory compounds such as antibacterial polyphenolics (TABASCO et al., 2011). Espírito-Santo et al. (2012) found a stimulatory effect of apple and banana flours (1%) on the viability of *L. acidophilus* L10 and *B. animalis* subsp. *lactis* Bl04, HN019 and B94 strains. The addition of 3% pea

flour to fermented milk also proved to be responsible for increasing the population of *L. rhamnosus* up to 0.95 log during storage (ZARE et al., 2013).

Table 3. *S. thermophilus, L. acidophilus* and *Bifidobacterium animalis* subsp. *lactis* populations (log CFU/mL) in fermented milk during storage.

Microorganisms	Days	Treatments			
		FMC	FMA	FMB	FMG
L. acidophilus	1	7.36 ± 0.12^{Ba}	7.53 ± 0.11^{Aa}	7.46 ± 0.09^{ABa}	7.56 ± 0.06^{Aa}
	14	7.30 ± 0.14^{Aa}	7.45 ± 0.11^{Aa}	7.38 ± 0.23^{Aa}	7.54 ± 0.08^{Aa}
	28	7.20 ± 0.05^{Ba}	7.38 ± 0.12^{Aa}	7.46 ± 0.07^{Aa}	7.27 ± 0.26^{ABb}
B. animalis	1	8.29 ± 0.17^{Ab}	8.37 ± 0.14^{Aa}	8.34 ± 0.17^{Aa}	8.41 ± 0.13^{Aa}
	14	7.57 ± 0.24^{Aab}	8.30 ± 0.21^{Ba}	8.30 ± 0.24^{Ba}	8.24 ± 0.19^{Ba}
	28	7.83 ± 0.08^{Aa}	8.08 ± 0.13^{Ba}	8.21 ± 0.15^{Ba}	7.95 ± 0.13^{Ba}
S. thermophilus	1	9.39 ± 0.06^{Aa}	9.30 ± 0.08^{Aa}	9.32 ± 0.12^{Aa}	9.35 ± 0.06^{Aa}
	14	9.51 ± 0.12^{Bb}	9.31 ± 0.06^{Aab}	9.24 ± 0.12^{Aa}	9.34 ± 0.08^{Aa}
	28	9.55 ± 0.04^{Bc}	9.37 ± 0.12^{ABb}	9.34 ± 0.14^{Aa}	9.24 ± 0.16^{ABb}

[a, b, c] Different lowercase letters denote significant differences ($P<0.05$) within the same treatment during storage. [A, B, C] Different capital letters denote significant differences ($P\leq0.05$) among treatments during the same storage period. FMC: fermented milk control, without addition of flour; FMA: fermented milk with addition of 1% apple flour; FMB: fermented milk with addition of 1% banana flour and FMG: fermented milk with addition of 1% grape flour. n = 3.

Even though the counts of probiotic strains decreased during the storage period, the minimum therapeutic effective dose, which has been suggested to be between 10^6–10^9 CFU in the product (VASILJEVIC; SHAH, 2008), was maintained in all treatments until 28 days of storage.

The population of *S. thermophilus* varied from 9.30 to 9.38 log CFU/mL after 1 day of cold storage, and the same counts were found ($P>0.05$) in all of the fermented milk samples (Table 3). However, on days 14 and 28, all supplemented fermented milk was found to have lower ($P\leq0.05$) counts of *S. thermophilus* compared to those of the control. Vinderola et al. (2002) noted the inhibition of *S. thermophilus* growth by some fruit juices (green apple, kiwi, pineapple, peach and strawberry) neutralized at pH 7.0.

3.5. Survival of *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 under simulated gastrointestinal conditions

In general, three results came from the *in vitro* assay: i) there was a significant reduction ($P \leq 0.05$) in the population of *B. animalis* subsp. *lactis* and *L. acidophilus* during the simulation of the *in vitro* gastrointestinal conditions; ii) there was a significant decrease ($P \leq 0.05$) in the population of the probiotics over storage time, because the cells were more stressed and damaged by the cold storage at the end of the storage period compared to the beginning. This behavior was also observed by Wang et al. (2009) and Vinderola et al. (2011); and iii) *B. animalis* subsp. *lactis* presented higher survival rates during the test than *L. acidophilus* did, and maintained mean populations of above 7 log CFU/mL up to the end of the assay in all of the products after all the storage test periods. The viability of *L. acidophilus* usually fell to below 4 log CFU/mL after the various stages of the simulated GI conditions (Figures 2 and 3).

Overall, the *L. acidophilus* population presented a considerable decrease during the assay (an average decrease between 1.99 and 4.34 log CFU/mL) in the 3 storage periods evaluated. At day 1, the presence of apple flour increased the population of *L. acidophilus* by no less than 1 log CFU/mL compared to control fermented milk. In the products with apple and banana flours, *L. acidophilus* cells were still recovered when the pH was increased to 7.0–7.3 after 6 h of assay. Similar results, which have been ascribed to a temporary damage of probiotic cells due to low pH stress, were observed by other authors in studies using other probiotic bacterial strains (CORSETTI et al., 2008; THANTSHA et al., 2009). The results suggest that this strain is highly sensitive toward simulated gastric juice containing HCl and pepsin, because the highest decrease in viability was observed during the gastric phase. After 14 days of refrigerated storage, the population of *L. acidophilus* continued to be higher in fermented milk with apple flour; however, the resistance to simulated GIT conditions was higher in the fermented milk with banana flour. In this period, there was a difference of 2 log CFU/mL between the banana fermented milk and the control sample at the end of the test. All of the flours had a positive effect on the survival of *L. acidophilus* compared to that of the control treatment when day 28 of refrigerated storage was reached.

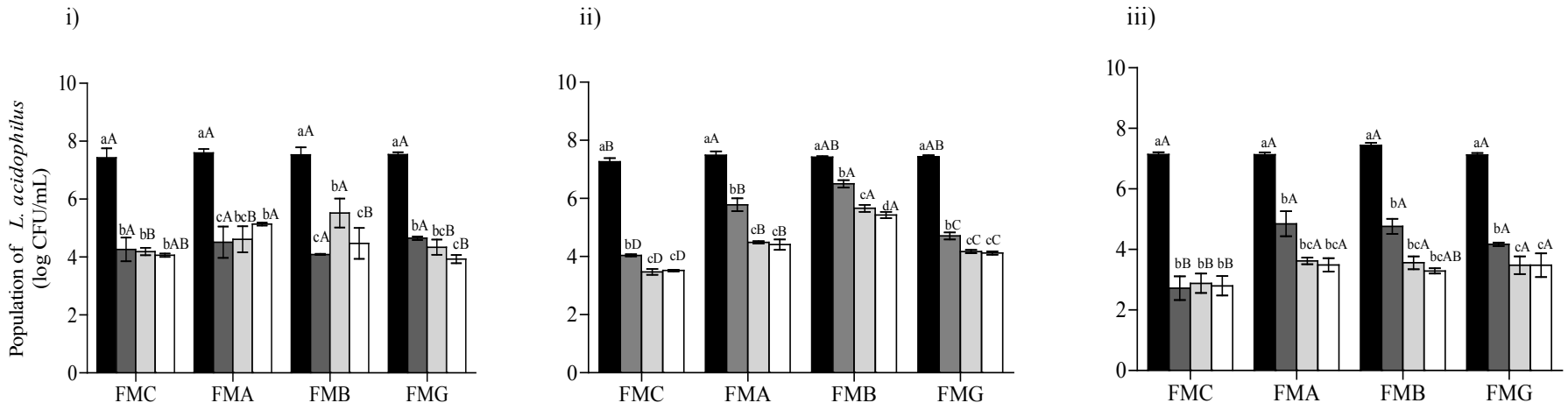

Figure 2. Survival of *L. acidophilus* La-5 (log CFU/mL) in fermented milks after 1, 14 and 28 days of storage (i, ii and iii, respectively), before (■) and during exposure to *in vitro* simulated gastric conditions, for 120 min (■, pH 2.0-2.2) and enteric conditions, for 240 (■, pH 4.3–5.2) and 360 (□, pH 7.0-7.3) min. [a, b, c] Different lower case letters denote significant differences ($P \leq 0.05$) among different sampling periods of the *in vitro* assay for the same treatment. [A, B, C] For the same storage period, different capital letters denote significant differences ($P \leq 0.05$) among treatments for the same sampling period of the *in vitro* assay. FMC (fermented milk control, without addition of flour), FMA (fermented milk added with 1% apple flour), FMB (fermented milk added with 1% banana flour) and FMG (fermented milk added with 1% grape flour). n = 3.

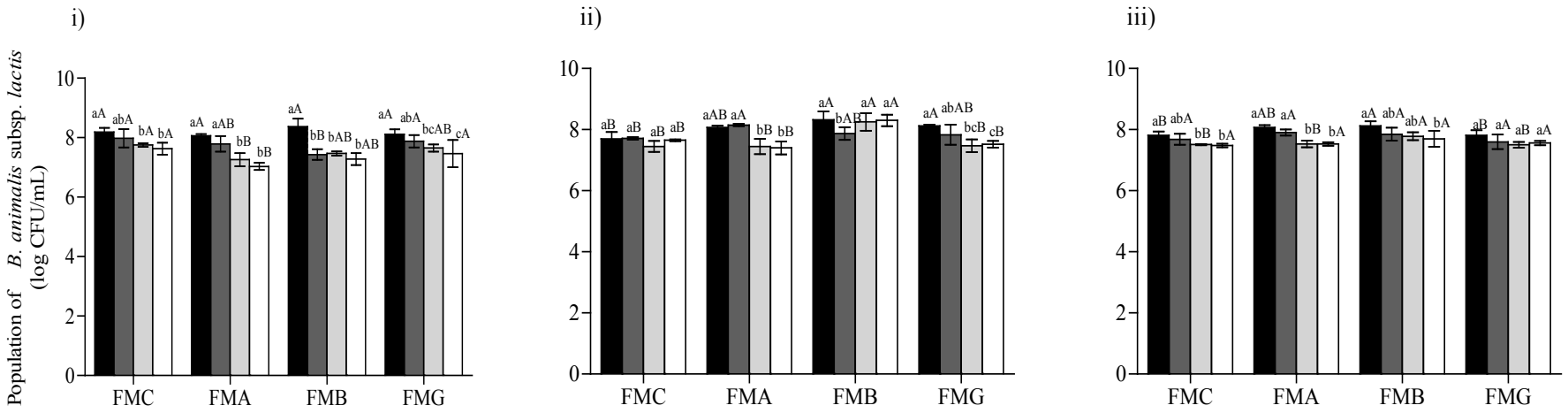

Figure 3. Survival of *B. animalis* subsp. *lactis* BB-12 (log CFU/mL) in fermented milks after 1, 14 and 28 days of storage (i, ii and iii, respectively), before (■) and during exposure to *in vitro* simulated gastric conditions, for 120 min (▬, pH 2.0-2.2) and enteric conditions, for 240 (▬, pH 4.3–5.2) and 360 (▭, pH 7.0-7.3) min. [a, b, c] Different lower case letters denote significant differences ($P \leq 0.05$) among different sampling periods of the *in vitro* assay for the same treatment. [A, B, C] For the same storage period, different capital letters denote significant differences ($P \leq 0.05$) among treatments for the same sampling period of the *in vitro* assay. FMC (fermented milk control, without addition of flour), FMA (fermented milk added with 1% apple flour), FMB (fermented milk added with 1% banana flour) and FMG (fermented milk added with 1% grape flour). n = 3.

The fruit flours improved *L. acidophilus* survival during the gastric phase (2 h), and also had a positive effect on *L. acidophilus* survivability during the enteric phase of the assay, especially on days 14 and 28 of storage. Additionally, the refrigerated storage period (28 days) was a determining factor for *L. acidophilus* survival, since the reduction of the *L. acidophilus* population from 0 to 6 h of assay was higher in all products on day 28 of storage.

A significant decrease ($P \leq 0.05$) in *L. acidophilus* was observed in most of the samples between the gastric and enteric phases, specifically after 14 and 28 days of refrigerated storage. This result suggests that this strain was also sensitive to conditions containing bile. Similar results were observed by Buriti; Castro and Saad (2010) and Bedani; Rossi and Saad (2013), using the same strain (*L. acidophilus* La-5) incorporated into guava mousse and fermented soy products, respectively. Bile primarily exerts its effects on cell membranes. It affects the phospholipids and proteins, and it disrupts cellular homeostasis. Gram-negative bacteria appear to be more inherently resistant to the deleterious effects of bile than Gram-positive bacteria do. In addition to affecting membrane characteristics, bile can have numerous other effects on bacterial cells, including disturbing macromolecule stability. Also, bile acids have been shown to induce secondary structure formation in RNA. However, tolerance to the bile is strain-specific and cannot be generalized (BEGLEY; GAHAN; HILL, 2005).

On day 1 of storage, the resistance of *B. animalis* subsp. *lactis* to simulated GIT conditions, in control and in fermented milk with grape flour, had a significantly higher viability ($P \leq 0.05$) than it did in fermented milk with apple and banana flours. On day 14, the best result was observed in the product with banana flour, and on day 28, there was no significant difference ($P \leq 0.05$) in the resistance of bifidobacteria among the treatments. The higher survivability of *Bifidobacterium* strains in simulated GIT conditions has been reported by other authors (MADUREIRA et al., 2011; BEDANI; ROSSI; SAAD, 2013). The improvement of *Bifidobacterium*'s intrinsic tolerance to gastrointestinal stress factors is a key element in guaranteeing the performance of probiotics in the intestine. An additional advantage of these bacteria is that they are able to accommodate their enzymatic system to the different challenges found along the GIT (SANCHEZ et al., 2013).

Despite the general definition that probiotics are live microorganisms, a variety of biological responses have been reported from administering dead, frequently heat-killed, probiotics to various mammalian and avian species. Live probiotic cells might

well influence the gastrointestinal microbiota and have an immunomodulating effect, whereas the components of dead cells could exert an anti-inflammatory response. However, the relative importance of these two effects is difficult to assess since an immunomodulating response of both live and dead probiotic cells has been extensively investigated. Dead probiotic cells are not a necessary requirement to generate a biological response but they may be sufficient (ADAMS et al., 2010).

The microenvironments produced by the food matrix or ingredient in the intestine may protect the probiotic microorganism from exposure to bile. Food constituents could bind to bile acids, preventing them from exerting their toxicity on the probiotics (BEGLEY; GAHAN; HILL, 2005). In this study, the fruit flours added to fermented milk are rich in proteins, lipids, and fibers. These components may have improved probiotic resistance to the simulated GI conditions. Even so, this effect was observed in the case of the *L. acidophilus* La-5 strain throughout the storage period and in the case of *B. animalis* subsp. *lactis* on day 14 of storage.

4. CONCLUSIONS

The addition of fruit flours did not influence the end time of fermentation, but it did affect V_{max} and t_{Vmax} and decrease post-acidification. The viability of *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 in all formulations of fermented milk was high through the 28th day of refrigerated storage, and it ranged from 7 to 8 log CFU/g in the case of both microorganisms. The addition of fruit flours did not improve the survival of probiotic strains in fermented milk samples during refrigerated storage. Resistance to simulated gastrointestinal conditions was high in the case of *B. animalis* subsp. *lactis* BB-12, and it kept mean populations above 7 or 8 log CFU/g at the end of the *in vitro* test. This value was lower in the case of *L. acidophilus* La-5 (below 5 log CFU/g). The presence of fruit flours protected *L. acidophilus* against the simulated gastrointestinal juices. Apple, banana and grape flours proved to be suitable for producing functional fermented milk.

ACKNOWLEDGMENTS

The authors would like to thank FAPESP (Sao Paulo Research Foundation, Sao Paulo, Brazil, grant 2009/12808-6) for the scholarship.

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Capítulo 4

Este trabalho foi submetido ao *International Journal of Food Microbiology*

EFFECT OF SUPPLEMENTING FERMENTED MILK WITH QUINOA FLOUR ON THE SURVIVAL OF PROBIOTIC BACTERIA EXPOSED TO SIMULATED GASTROINTESTINAL TRANSIT AND ON THEIR ADHESION TO EPITHELIAL CELLS

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ABSTRACT

The viability of probiotic microorganisms in the gut is one of the most important challenges faced by researchers. In this study, functional fermented milk was produced using the ABT-4 culture (*S. thermophilus, L. acidophilus* and *B. animalis* subsp. *lactis*) supplemented with quinoa flour (1-3%). The aim was to evaluate the effect this supplementation by evaluating the kinetics of acidification, the pH and titratable acidity, bacterial viability, the resistance of probiotics to simulated gastrointestinal (GI) conditions and the adhesion of probiotics to Caco-2 cells *in vitro*. The results for t_{Vmax}, $t_{pH5.0}$ and $t_{pH4.6}$ were similar for all treatments. Therefore, adding quinoa flour had no effect on the fermentation time; however, it contributed to post-acidification of the fermented milk during storage. Quinoa flour did not affected the counts of *B. animalis* subsp. *lactis* and *L. acidophilus* during the storage, did not protected the probiotic strains during simulated GI transit and did not have a positive effect on the adhesion of probiotic bacteria to Caco-2 cells *in vitro*, because the differences found between control treatment and supplemented treatments were below 1 log UFC/mL. Addicionally, the adhesion of strains to Caco-2 cells reduced during the refrigerated storage of fermented milk.

Key words: functional food, fermented dairy products, probiotics, gastrointestinal resistance, Caco-2 adhesion.

1. INTRODUCTION

Worldwide, the demand for functional foods is growing rapidly due to increased consumer awareness of the impact of food on health. Functional foods not only provide nutrients but also contain biologically active components that can improve health and well-being, reducing the risk of disease by beneficially affecting one or more target functions in the body (Mohammadi and Mortazavian, 2011). Probiotic bacteria belong to this category of functional foods. The global market for ingredients, food and probiotic supplements are worth $14.9 billion USD in 2007 and $16 billion USD in 2008. By 2013, total sales of probiotic products are estimated to reach US $19.6 billion, and foods with probiotic bacteria continue to be of considerable economic interest (Granato et al., 2010a).

Probiotics are live microorganisms that confer benefits to the host when consumed in adequate amounts (FAO/WHO, 2002). Reports indicate that probiotics exert beneficial effects on the immune system and the gut, reduce side effects associated with antibiotic use, reduce symptoms associated with irritable bowel syndrome, help alleviate lactose intolerance, and have antimicrobial and anticancer properties (Fontana et al., 2013). In the current market scenario, dairy products such as yogurt, fermented milk and cheese dominate the probiotic food sector (Madhu et al., 2012). It is widely accepted that probiotics must be present at a minimum level, ranging from 10^6 to 10^9 CFU/mL of the product, to be beneficial. The best-known probiotic microorganisms include strains within the *Lactobacillus* and *Bifidobacterium* genera (Vasiljevic and Shah, 2008).

Many ingredients can be added to fermented milk products to enhance their nutritional value and stimulate probiotic growth and activity during cold storage. These additives can keep the population of probiotics above the minimum recommended dose and meet consumer needs for new products. In addition, these ingredients may also help probiotics survive during passage through the gastrointestinal tract by increasing their resistance to gastric and enteric juices (Bedani et al., 2013; Hernandez-Hernandez et al., 2012).

The potential of quinoa to provide the above-mentioned benefits is still unknown. Quinoa is a pseudocereal originally from Andean region, which is home to one of the oldest cultures in the Americas. This grain is still grown in Peru, Bolivia,

Chile, Ecuador, Colombia and Argentina and has recently been introduced in Europe, North America, Africa and Asia (Bhargava et al., 2006). Compared to other cereals, quinoa has higher protein content (16%) with a balanced amino acid composition and higher amounts of lysine (5.8%) and methionine (2.4 to 5.1%). Quinoa is also rich in dietary fiber, nutrients and a wide range of vitamins. Its antioxidant capacity is associated with its content of phenolic compounds, such as α-tocopherol (vitamin E) (Fischer et al., 2013). Quinoa flour contains approximately 7% of dietary fiber and is rich in unsaturated fatty acids (Hager et al., 2012). Also, the biological effects of supplementation with hydrolyzed quinoa extract have been demonstrated *in vivo*. Supplementation with hydrolyzed quinoa decreased body weight gain, food intake, fat deposition, and triacylglycerol levels in sedentary and exercised rats, suggesting a potential use in human nutrition (Meneguetti et al., 2011).

In this study, we investigated the effects of supplementing milk with 1-3% quinoa flour on kinetics of acidification during fermentation and changes in post-acidification, viable counts of *Streptococcus thermophilus* and probiotic strains (*Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus*), resistance of probiotics to simulated gastrointestinal conditions and *in vitro* adhesion of probiotics to intestinal cells during 28 days of refrigerated storage in fermented milk.

2. MATERIAL AND METHODS

2.1. Culture and ingredients

Skimmed milk powder (SMP) (Nestlé, Araçatuba, SP, Brazil), ABT-4 culture (Chr. Hansen, Valinhos, SP, Brasil), which is composed of *Streptoccus thermophilus, Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12, and commercial quinoa flour (Sun Products do Brasil Ind. Com. Ltda., Jaraguá do Sul, SC, Brazil) were used in this study. According to the manufacturer, the quinoa flour is composed of 74.3% carbohydrates, 13.5% protein, 4.9% fat and 5.1% dietary fiber.

2.2. Inoculum preparation

The ABT-4 pre-culture was suspended in amounts recommended by the manufacturer in 50 mL of sterile milk and activated at 42 °C for 30 min. The initial

colony counts after milk inoculation were approximately 10^6 CFU/mL for *L. acidophilus* La-5 and 10^7 CFU/mL for *S. thermophilus* and *B. animalis* subsp. *lactis* BB-12.

2.3. Milk base preparation

Four different milk bases were prepared: milk supplemented with quinoa flour at concentrations of 1 (FMQ1), 2 (FMQ2) and 3% (FMQ3) (w/v) and milk without quinoa flour (control - FMC). Skim milk powder (Nestlé, Araçatuba, SP, Brazil) was reconstituted in water to 13 g/100 g of total solids. All the milk bases were heated at 90 °C for 10 min in water bath and transferred to 500 mL-sterile Schott flasks inside a laminar flow chamber, and stored at 4 °C for 24 h before use.

2.4. Fermentations and kinetic parameters

Before fermentation, milk (500 mL) was heated to 42 °C and 2 mL of the pre-culture was added. After inoculation, the flaskss were transferred to a water bath connected to a CINAC (Cynetique d´acidificacion, Alliance Instruments, Frepillon, France) system that continuously measures pH, allowing the evaluation of the acidification rate throughout the fermentation process. Batch fermentations were performed in three independent trials at 42 °C until the pH reached 4.6. From the collected data, the acidification rate (V_{max}) was calculated as the change in pH over time (dpH/dt) and expressed as 10^{-3} pH units/min. During the incubation period, the following kinetic parameters were also calculated: (i) t_{Vmax} (h, time at which V_{max} was reached), (ii) $t_{pH5.0}$ (h, time to reach pH 5.0), (iii) $t_{pH4.6}$ (h, time to reach pH 4.6, i.e., time to complete the fermentation).

At the end of fermentation, the fermented milk was cooled to 15 °C in an ice bath, and then the clot was broken up with a stainless steel perforated disk with up and down movements for approximately 1 min. The product was packed in 80-mL sterile plastic cups and was stored at 4 °C for 28 days.

2.5. Post-acidification and titratable acidity

The post-acidification of the fermented milk was measured 1, 14 and 28 days post-storage at 4 °C by measuring the pH in triplicate using a pHmeter model PG1800 (Gehaka, São Paulo, Brazil). Acidity, expressed as % of lactic acid per 100 grams, was determined in triplicate for each trial by titration with 0.1 N NaOH solution (AOAC, 2005).

2.6. Microbiological analyses

Plate counts for each treatment were done in duplicate after 1, 14 and 28 days of storage. Colony counts for *S. thermophilus, L. acidophilus and B. animalis* subsp. *lactis* were performed using M17 agar (Himedia, Mumbai, India), MRS agar (Acumedia, Lansing, MI, USA) with added bile solution (Sigma-Aldrich, St. Louis, MO, USA) and MRS agar (Acumedia, Lansing, MI, USA) with added 0.2% lithium chloride and 0.3 % sodium propionate (Sigma-Aldrich, St. Louis, MO, USA), respectively. M17, MRS-bile and MRS-LP media were prepared according to IDF (1997), IDF (1995) and Vinderola and Reinheimer (1999), respectively. *S. thermophilus* and *L. acidophilus* plates were incubated at 37 °C for 48 h under aerobic conditions. Plates of *B. animalis* subsp. *lactis* were incubated at 37 °C for 72 h under anaerobic conditions provided by Anaerobac (Probac, São Paulo, Brazil). The results were expressed as log CFU/mL of fermented milk.

2.7. Probiotic survival under conditions that simulate the gastrointestinal tract

The survival of probiotic bacteria in fermented milk submitted to simulated GI conditions was evaluated according to the method described by Buriti, Castro and Saad (2010), with modifications, after 1, 14 and 28 days of refrigerated storage. Briefly, the fermented milk samples were diluted in 0.5% NaCl, and 10 mL was transferred to three sterile flasks. The pH was adjusted to 2.0–2.2 with 0.5 N HCl, and pepsin (3 g/L) (from porcine stomach mucosa, Sigma-Aldrich, St. Louis, MO, USA) and lipase (0.9 mg/L) (Amano lipase F-AP15 from *Rhyzopus oryzae*, Aldrich Chemical Company, Milwaukee, WI, USA) solutions were added to the samples. The flasks were then incubated at 37 °C for 2 h (gastric phase). Next, the sample pH was increased to 4.3–5.2 using an alkaline solution containing bile (10 g/L) (bovine bile, Sigma-Aldrich, St.

Louis, USA) and pancreatin (1 g/L) (pancreatin from porcine pancreas, Sigma-Aldrich, St. Louis, MO, USA), and the flasks were incubated again at 37 °C for 2 h (enteric phase 1). Finally, the pH was increased to 7.0–7.3 using the same alkaline solution, bile and pancreatin concentrations were adjusted (10 g/L and 1 g/L, respectively), and samples were incubated again at 37 °C for 2 h (enteric phase 2). Aliquots of 1 mL were collected from duplicate samples after 0, 2, 4 and 6 h in order to assess gastrointestinal transit tolerance of probiotic bacteria. The media and growth conditions employed were the same as described in Section 2.6. The experiment was performed in duplicates.

2.8. Adhesion to Caco-2 cells

The Caco-2 cell line ATCC HTB-37 (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) was routinely cultured (passages 29-31) in Dubelcco's modified Eagle's minimum (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 20% heat-inactivated fetal bovine serum (Cultilab, Campinas, SP, Brazil), a mixture of penicillin (100 U.I./mL) and streptomycin (100 µg/mL) (Sigma Aldrich, St. Louis, MO, USA), and 1% non-essential amino acid solution (Sigma Aldrich, St. Louis, MO, USA), at 37 °C, in an atmosphere of 5% CO_2. The adhesion assay was performed as described by Ranadheera et al. (2012), with modifications. Briefly, Caco-2 cells were seeded at a concentration of 10^5 cells/well into 24-well tissue culture plates (Nest Biotechnology Co. Ltda., Wuri, China) and incubated at 37 °C in an atmosphere of 5% CO_2 (Thermo Electrone, Sunnyvale, CA, USA) until a confluent monolayer had formed (15-17 days). One day before the adhesion assays, the medium was replaced with the same medium but without antibiotics. Before adhesion, the monolayer was washed once with phosphate-buffered solution (PBS, pH 7.4), to remove all traces of the medium. An aliquot of 1 mL of each fermented milk sample was transferred to post-confluent monolayers of Caco-2 cells in 24-well tissue culture plates and incubated at 37 °C in a 5% CO_2 atmosphere for 2 h. Cells were then washed at least three times with PBS to remove non-adherent bacteria and detached from each well by the addition of 1 mL of Triton X-100 (0.5% v/v) (Sigma Aldrich, St. Louis, MO, USA). The suspension (1 mL) from each well was then transferred to a tube containing 9 mL of peptone water, serially diluted, and plated on the appropriate media (Section 2.6) in duplicate to determine adhesion ability. Percent adhesion was calculated by determining the viable bacterial counts of the fermented milk before the adhesion assay and viable bacterial counts

adhered to the cell layers from each type of fermented milk. The experiment was performed in duplicate.

2.9. Statistical analyses

Results were analyzed by two-way ANOVA using STAT Software version 2.0. Mean values were compared using the Tukey test at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Kinetic parameters of acidification

The acidification profiles of the fermented milk control and supplemented with quinoa flour (1-3%) were evaluated using the parameters V_{max}, t_{Vmax}, pH_{Vmax}, $t_{pH5.0}$ and $t_{pH4.6}$ (Table 1).

Table 1. Kinetic parameters of acidification of the fermented milk control and fermented milk supplemented with quinoa flour at different concentrations.

Treatments	V_{max} (10^{-3} upH/min)	t_{Vmax} (h)	pH_{Vmax}	$t_{pH5.0}$ (h)	$t_{pH4.6}$ (h)
FMC	23.40±0.84^{a}	2.08±0.11^{a}	5.73±0.03^{a}	2.97±0.28^{a}	4.54±0.52^{a}
FMQ1	20.08±1.57^{ab}	2.15±0.21^{a}	5.59±0.03^{b}	2.92±0.35^{a}	4.42±0.49^{a}
FMQ2	18.17±0.15^{b}	2.17±0.19^{a}	5.57±0.01^{bc}	2.95±0.30^{a}	4.53±0.57^{a}
FMQ3	17.27±0.06^{b}	2.20±0.14^{a}	5.46±0.05^{c}	2.90±0.18^{a}	4.54±0.37^{a}

$^{a, b, c}$ Different letters in the same column denotes significant difference ($P \leq 0.05$) among treatments. FMC (fermented milk control, without quinoa flour), FMQ1 (fermented milk supplemented with 1% quinoa flour), FMQ2 (fermented milk supplemented with 2% quinoa flour) and FMQ3 (fermented milk supplemented with 3% quinoa flour). V_{max} = maximum acidification rate; t_{Vmax} = time to reach V_{max}; pH_{Vmax} = pH in V_{max}; $t_{pH5.0}$ = time to reach pH 5.0; $t_{pH4.6}$ = time to reach pH 4.6 (end of fermentation). n = 3.

V_{max} was significantly ($P \leq 0.05$) higher in the fermented milk control without quinoa flour and in the fermented milk supplemented with 1% quinoa flour. The pH_{Vmax} was also significantly different ($P \leq 0.05$) among the treatments. The buffering capacity of the fermented milk product is directly proportional to its total solids content, which

can result in higher kinetic parameters and longer fermentation (Varghese and Mishra, 2008). In this study, the addition of higher quinoa flour concentrations (2 and 3%) resulted in lower V_{max} values. Therefore, the addition of this ingredient decreases the ability of the ABT-4 microorganisms to acidify the milk. An inverse relationship between V_{max} and t_{Vmax} was observed: lower V_{max} corresponded to longer times required to reach the V_{max}. However, the results for t_{Vmax}, $t_{pH5.0}$ and $t_{pH4.6}$ were statistically (P>0.05) equal for all treatments (Table 1). In this study, the addition of quinoa flour had no effect (P>0.05) on the fermentation time of products, which is good from an industrial standpoint.

3.2. Post-acidification and titratable acidity

Throughout the study, significant differences (P>0.05) in pH reduction and in titratable acidity increase were observed for all products (Table 2). However, acid production was low after 28 days, and the products had acidities typical for commercial products. According to current Brazilian legislation, fermented milk acidity must range from 0.6 to 2% of lactic acid per 100 g. The consumption of residual lactose and production of lactic acid occurs during the storage of fermented milk as a result of the metabolic activity of lactic acid bacteria (a phenomenon known as post-acidification) (Oliveira et al., 2009).

Fermented milk with quinoa flour exhibited significantly lower titratable acidity values (P≤0.05) than the control treatment at all time points, and the pH value was significantly different (P≤0.05) between treatments only after 28 days of storage, when the fermented milk control showed the highest pH and samples fermented with 2 and 3% quinoa flour had the lowest pH values. The pH decreased from 0.28 to 0.38 units during the 28 days of storage, but the reduction was higher in samples supplemented with quinoa flour than in the control sample. The acidity increased from 0.24 to 0.27% in fermented milk during storage. The microorganisms from the ABT-4 starter culture likely have the ability to hydrolyze the carbohydrates in quinoa flour and converted it into organic acids, though this ingredient does not stimulate microorganism growth during fermentation. Likewise, Zare et al. (2012) found that fermented milk supplemented with 1-3% pea flour exhibit a higher reduction in pH during the storage than the control product. However, other studies reported that the addition of fibers (apple, wheat, bamboo, inulin, orange, lemon, date and wheat bran) did not affect the

pH and acidity of yogurts (Hashim et al., 2009; Sendra et al., 2008; Staffolo et al., 2004). Thus, post-acidification seems to be dependent upon the type of starter culture and ingredients in milk bases.

Table 2. Titratable acidity and post-acidification (pH) of fermented milk control and fermented milk supplemented with quinoa flour during refrigerated storage.

Treatments	Storage (days)		
	1	14	28
Titratable acidity			
FMC	0.82 ± 0.04 ^{Bc}	1.03 ± 0.01 ^{Ab}	1.06 ± 0.01 ^{Ab}
FMQ1	0.87 ± 0.04 ^{Cb}	1.08 ± 0.01 ^{Ba}	1.14 ± 0.04 ^{Aa}
FMQ2	0.88 ± 0.01 ^{Cb}	1.09 ± 0.01 ^{Ba}	1.14 ± 0.04 ^{Aa}
FMQ3	0.94 ± 0.04 ^{Ca}	1.09 ± 0.01 ^{Ba}	1.14 ± 0.03 ^{Aa}
pH			
FMC	4.59 ± 0.03 ^{Aa}	4.31 ± 0.01 ^{Ba}	4.31 ± 0.03 ^{Ba}
FMQ1	4.63 ± 0.03 ^{Aa}	4.30 ± 0.06 ^{Ba}	4.27 ± 0.05 ^{Bab}
FMQ2	4.64 ± 0.02 ^{Aa}	4.28 ± 0.04 ^{Ba}	4.25 ± 0.03 ^{Bb}
FMQ3	4.63 ± 0.03 ^{Aa}	4.29 ± 0.06 ^{Ba}	4.25 ± 0.01 ^{Bb}

Values are expressed as the mean ± SD. FMC (fermented milk control, without quinoa flour), FMQ1 (fermented milk supplemented with 1% of quinoa flour), FMQ2 (fermented milk supplemented with 2% quinoa flour) and FMQ3 (fermented milk supplemented with 3% quinoa flour). $^{A, B, C}$ Different capital letters in a row denote significant differences for the same treatment during storage period ($P \leq 0.05$). $^{a, b, c}$ Different lowercase letters in a column denote significant differences among treatments ($P \leq 0.05$). n = 3.

The largest decrease in acidity and increase in pH occurred between the 1st and 14th days because the microorganisms have high metabolic activity at higher pH, which occurs during the beginning of the storage, as observed by Beal et al. (1999).

3.3. Microorganisms viability

The inoculation rates of *S. thermophilus* and probiotic strains in milk before fermentation showed no significant difference ($P > 0.05$) between treatments (data not shown). During the storage period, the population of *L. acidophilus* ranged from 7.04 to 7.94 log CFU/mL (Table 3), showing a reduction from 0.17 to 0.43 log CFU/mL at the

end of storage. The population of *B. animalis* subsp. *lactis* ranged from 7.61 to 8.49 log CFU/mL during 28 days of storage (Table 3), reducing from 0.10 to 0.49 log CFU/mL. For both probiotic bacteria, the largest decreases at the end of storage were obtained for treatments without quinoa flour. Thus, supplementing milk with 1-3% quinoa flour resulted in a significantly ($P \leq 0.05$) higher population of *L. acidophilus* and *B. animalis* subsp. *lactis* compared to the control treatment after 28 days of storage. However, these changes were of little microbiological significance, since they were below 1 log CFU/mL. Nervertheless, the addition of 3% quinoa flour showed a tendency towards stimulation of *B. animalis* subsp. *lactis* during storage. In this case, the population of this strain was 0.87 log CFU/mL greater than in the fermented milk control. These results are similar to those previously observed for fermented milk with lentils, pea and orange fiber (Agil et al., 2013; Sendra et al., 2008; Zare et al., 2013).

The improvement of this probiotic strain growth in the presence of 3% quinoa flour could be related to the compounds present in this ingredient. Quinoa flour is characterized by high carbohydrate and fiber content, relatively high in linoleic acid (52.68% of total fatty acid content), contributing to 26% and 37% (male and female) of the dietary reference intake. It is also rich in oleic (23.93% of total fatty acid content) and α-linolenic (4.60% of total fatty acid content) acids. Folate, potassium, phosphorous, magnesium and calcium contents are also high (180 μg/100 g, 553.8, 441.6, 229.9 and 49.8 mg/100 g, respectively). Additionally, quinoa flour is high in iron and zinc (5.4 and 3.3 mg/100 g respectively) (Hager et al., 2012). Because probiotic microorganisms require high nutrient levels, these compounds benefit the growth of probiotics during storage of fermented milk. However, further studies are necessary to elucidate which specific components are involved in improving the growth and survival of probiotics in products supplemented with quinoa flour.

An agreement on the amount of probiotic bacteria that should be present in food to ensure the health beneficial effects has not been reached, but there are several proposals for the minimum number of viable bacteria (Zare et al., 2012). Depending on the type of food or probiotic strain involved, the minimum probiotic strain concentration in fermented products is suggested to be between 10^6-10^9 UFC/g or mL (Granato et al., 2010b). Moreover, it is important to monitor the viability of the probiotic during manufacture and storage to maintain these levels. Despite the variations observed in the probiotic populations, the minimum therapeutic dose, suggested between 10^6-10^9

CFU/mL in the product (Vasiljevic and Shah, 2008), was achieved in all treatments until day 28.

Table 3. *S. thermophilus, L. acidophilus* and *Bifidobacterium animalis* subsp. *lactis* populations (log CFU/mL) in fermented milk during storage.

Microorganisms	Days	Treatments			
		FMC	FMA	FMB	FMG
L. acidophilus	1	7.47 ± 0.03^{Ab}	7.73 ± 0.19^{ABa}	7.71 ± 0.12^{Bab}	7.83 ± 0.09^{Aa}
	14	7.43 ± 0.11^{Ab}	7.86 ± 0.09^{Aa}	7.94 ± 0.06^{Aa}	7.91 ± 0.08^{Aa}
	28	7.04 ± 0.02^{Bb}	7.56 ± 0.05^{Ba}	7.51 ± 0.10^{Ca}	7.63 ± 0.08^{Ba}
B. animalis	1	8.10 ± 0.27^{Ab}	8.46 ± 0.06^{Aa}	8.44 ± 0.07^{Aa}	8.49 ± 0.05^{Aa}
	14	7.93 ± 0.09^{ABb}	8.47 ± 0.12^{Aa}	8.47 ± 0.02^{Aa}	8.45 ± 0.06^{Aa}
	28	7.61 ± 0.06^{Bc}	8.36 ± 0.04^{Ab}	8.40 ± 0.05^{Aab}	8.48 ± 0.05^{Aa}
S. thermophilus	1	9.41 ± 0.23^{Aa}	9.43 ± 0.02^{ABa}	9.42 ± 0.13^{Aa}	9.58 ± 0.26^{Aa}
	14	9.49 ± 0.09^{Aa}	9.53 ± 0.09^{Aa}	9.31 ± 0.06^{Ab}	9.29 ± 0.03^{ABb}
	28	9.28 ± 0.09^{Aa}	9.30 ± 0.12^{Aa}	9.25 ± 0.07^{Aa}	9.23 ± 0.06^{Ba}

$^{A, B, C}$ Different capital letters denote significant differences ($P\leq0.05$) for the same treatment during storage. $^{a, b, c}$ Different lowercase letters denote significant differences ($P\leq0.05$) among treatments for the same storage period. FMC: fermented milk control, without quinoa flour; FMQ1: fermented milk supplemented with 1% quinoa flour; FMQ2: fermented milk supplemented with 2% quinoa flour; and FMQ3: fermented milk supplemented with 3% quinoa flour. n = 3.

In addition, a positive correlation was observed between the population of probiotic strains (r = 0.87 and r = 0.95 for La-5 and BB-12, respectively) and the pH value of the samples in the 1st day of storage. However, at the end of storage, a negative correlation was observed between these parameters (r = -0.94 and r = -0.96, for La-5 and BB-12, respectively). These data are in agreement with other studies showing that the acidification during storage does not necessarily correspond to kinetics of microbial growth (Seo et al., 2009; Zare et al., 2013).

The population of *S. thermophilus* ranged from 9.23 to 9.58 log CFU/mL during storage of the products (Table 3). The variations found in the population, both over time and among treatments are minimal. Several studies have demonstrated the stability of the population of *S. thermophilus* during food product storage (Bedani et al., 2013; Madhu et al., 2012).

3.4. Probiotic resistance to simulated gastrointestinal conditions

At the beginning of the *in vitro* assay, the populations of *L. acidophilus* and *B. animalis* subsp. *lactis* were higher than 7 log CFU/mL and 8 log CFU/mL respectively, which meet the recommended amount for probiotics, for all treatments and for all days of analysis. However, there was a significant reduction ($P \leq 0.05$) in the population of probiotic bacteria during the passage through the simulated conditions of the gastrointestinal tract. The protective effect of quinoa flour over probiotic strains resistance against simulated GI conditions was not significant from the microbiological standpoint, since the difference between the probiotic counts in control and in supplemented fermented milk at the end of the *in vitro* assay was smaller than 1 log CFU/mL. The reduction of the population after the assay was influenced by the storage period (Table 4).

Probiotic strains must reach the intestine in sufficient concentration to exert their beneficial effects. Therefore, an ideal carrier must be suitable for human consumption, ensure the viability of the strains during the manufacturing time and shelf-life of the product and protect the probiotics during passage through the gastrointestinal tract so that they can reach the colon (Possemiers et al., 2010).

Recent studies have reported that food matrix and dietary content structures may interact with probiotic strains, protecting them during transit through the gastrointestinal tract (Lavermicocca, 2006; Valerio et al., 2006). The presence of quinoa flour may have provided some protection for the survival of probiotics during the simulated passage through the gastrointestinal tract by acting as a protective cover against the gastric and enteric juices. Therefore, it is possible that flour components (protein, lipids and fiber) improved probiotic tolerance to simulated GI conditions. Nevertheless, this effect was only pronounced for *L. acidophilus* against gastric juice action on the last day of storage and at the end of the *in vitro* assay on the 14th day of storage.

Table 4. Probiotic survival (log CFU/mL) in fermented milk after 1 (D1), 14 (D14) and 28 (D28) days of storage, before (t0) and during exposure to *in vitro* simulated gastric conditions, for 120 min (t120, pH 2.0-2.2), and enteric conditions, for 240 min (t240, pH 4.3-5.2) and 360 min (t360, pH 7.0-7.3).

Strain	Treatment	Days	Viable counts (log CFU/mL) during simulated GIT conditions			
			t0	t120	t240	t360
La-5	FMC	D1	7.45±0.07 ^{Ab}	4.76±0.08 ^{Bc}	4.16±0.08 ^{Db}	4.36±0.07 ^{Cc}
	FMQ1	D1	7.78±0.16 ^{Aa}	5.20±0.04 ^{Bb}	4.79±0.04 ^{Ca}	4.57±0.02 ^{Db}
	FMQ2	D1	7.84±0.13 ^{Aa}	5.70±0.06 ^{Ba}	4.87±0.06 ^{Ca}	4.77±0.04 ^{Ca}
	FMQ3	D1	7.93±0.04 ^{Aa}	5.80±0.17 ^{Ba}	4.96±0.16 ^{Ca}	4.84±0.01 ^{Ca}
	FMC	D14	7.32±0.13 ^{Ab}	4.47±0.04 ^{Bb}	3.23±0.08 ^{Cc}	3.43±0.13 ^{Cc}
	FMQ1	D14	7.77±0.12 ^{Aa}	5.27±0.08 ^{Ba}	4.38±0.22 ^{Cb}	4.12±0.27 ^{Db}
	FMQ2	D14	7.99±0.06 ^{Aa}	5.07±0.23 ^{Ba}	4.68±0.10 ^{Ca}	4.60±0.03 ^{Ca}
	FMQ3	D14	7.79±0.17 ^{Aa}	5.30±0.07 ^{Ba}	4.44±0.19 ^{Cab}	4.69±0.09 ^{Ca}
	FMC	D28	7.08±0.07 ^{Ab}	2.77±0.25 ^{Bc}	2.53±0.14 ^{Bb}	2.52±0.05 ^{Bc}
	FMQ1	D28	7.66±0.06 ^{Aa}	3.91±0.22 ^{Bab}	3.07±0.06 ^{Ca}	2.82±0.14 ^{Db}
	FMQ2	D28	7.48±0.06 ^{Aa}	3.79±0.12 ^{Bb}	3.04±0.10 ^{Ca}	3.19±0.02 ^{Ca}
	FMQ3	D28	7.70±0.03 ^{Aa}	4.10±0.29 ^{Ba}	3.21±0.09 ^{Ca}	3.23±0.02 ^{Ca}
BB-12	FMC	D1	8.00±0.03 ^{Ac}	7.97±0.06 ^{Ac}	7.76±0.08 ^{Bc}	7.66±0.05 ^{Bc}
	FMQ1	D1	8.33±0.03 ^{Ab}	8.20±0.10 ^{Ab}	7.92±0.13 ^{Bb}	7.92±0.13 ^{Bb}
	FMQ2	D1	8.54±0.81 ^{Aa}	8.47±0.03 ^{Aa}	7.94±0.02 ^{Bb}	7.94±0.04 ^{Bb}
	FMQ3	D1	8.52±0.09 ^{Aa}	8.40±0.04 ^{Aa}	8.07±0.03 ^{Ca}	8.20±0.09 ^{Ba}
	FMC	D14	8.06±0.08 ^{Ab}	8.03±0.03 ^{Ab}	7.87±0.06 ^{Bb}	7.79±0.06 ^{Bb}
	FMQ1	D14	8.37±0.13 ^{Aa}	8.38±0.03 ^{Aa}	8.03±0.05 ^{Ba}	8.03±0.07 ^{Ba}
	FMQ2	D14	8.47±0.06 ^{Aa}	8.42±0.03 ^{Aa}	7.97±0.09 ^{Cab}	8.17±0.06 ^{Ba}
	FMQ3	D14	8.46±0.06 ^{Aa}	8.34±0.04 ^{Aa}	8.03±0.02 ^{Ba}	8.14±0.17 ^{Ba}
	FMC	D28	7.78±0.07 ^{Ab}	7.74±0.15 ^{Ab}	7.56±0.06 ^{Bb}	7.18±0.19 ^{Cb}
	FMQ1	D28	8.41±0.07 ^{Aa}	8.26±0.04 ^{Aa}	7.91±0.07 ^{Ba}	8.02±0.01 ^{Ba}
	FMQ2	D28	8.36±0.10 ^{Aa}	8.27±0.08 ^{Aa}	7.84±0.03 ^{Ca}	8.01±0.01 ^{Ba}
	FMQ3	D28	8.49±0.08 ^{Aa}	8.31±0.13 ^{Ba}	7.87±0.00 ^{Ca}	8.05±0.07 ^{Da}

$^{A, B, C}$ Different capital letters in the same row denote significant differences ($P \leq 0,05$) among sampling period of the *in vitro* assay at each day of storage for the same treatment. $^{a, b, c}$ Different lower case letters in the same column denote significant differences ($P \leq 0,05$) among treatments at each sampling day for each probiotic strain. FMC (fermented milk control, without quinoa flour), FMQ1 (fermented milk supplemented with 1% quinoa flour), FMQ2 (fermented milk supplemented with 2% quinoa flour) and FMQ3 (fermented milk supplemented with 3% quinoa flour). n = 3.

In general, the viability of *L. acidophilus* at the end of the *in vitro* assay dropped 3 log cycles on day 1 for all treatments, 4 log cycles on day 14 for FMC treatment and 3 log cycles for the other treatments, and 4 log cycles on day 28 for all treatments. The gastric phase showed a greater deleterious effect on *L. acidophilus* cells than the enteric phase. In general, the acid tolerance of LAB depends on the pH profile of their H^+-ATPase enzyme, coupled with their cytoplasmic membrane, which depends largely on the species of bacterium, as well as on exogenous conditions, such as media type of growth medium and incubation conditions (Madureira et al., 2011).

B. animalis subsp. *lactis* was more resistant than lactobacilli to the GI conditions. Some strains of *Bifidobacterium* spp. have been suggested to possess acid stress adaptation strategies, and the behavior of this microorganism during exposure to low pH may be strain dependent. Most strains resistant to gastric juice belong to the species *B. animalis.* When these strains are exposed to artificial gastric juice, characterized by low pH and the presence of pepsin, survival is greater than when exposed only to a low pH value, as pepsin somehow protects bacterial cells. Pepsin helps maintain pH homeostasis in bifidobacteria cells and supports the role of H^+-ATPase in the protective ability of pepsin in this species (Mättö et al., 2006).

The impact of pancreatin on the viability of probiotics is relatively low (Champagne and Gardner, 2008; Ruiz-Moyano et al., 2008). Thus, probiotic death during exposure to enteric phase occurs mainly by the action of bile salts. Bile salts are natural detergents that facilitate the digestion and absorption of hydrophobic components of the diet and bile antimicrobial nature arises primarily from its detergent property, which dissolves the bacterial membranes. Their amphiphilic nature promotes stronger inhibition of bacteria, thus making their survival more difficult throughout the gastrointestinal tract (Madureira et al., 2011). However, for both strains tested in all treatments, there was little variation in viable cell numbers during the enteric phase, which can be explained by the neutral pH prevailing during that period. *In vivo*, if the cells reached this point, they would most likely adhere to the intestinal epithelium and begin to exert their beneficial biological activities.

Despite the general definition that the terapeutic effects of probiotic is promoted by the comsumption of live microorganisms, a variety of biological responses have been reported from administering dead, frequently heat-killed, probiotics to various mammalian and avian species. Live probiotic cells might well influence the gastrointestinal microbiota and have an immunomodulating effect, whereas the

components of dead cells could exert an anti-inflammatory response. However, the relative importance of these two effects is difficult to assess since an immunomodulating response of both live and dead probiotic cells has been extensively investigated. Dead probiotic cells are not a necessary requirement to generate a biological response but they may be sufficient (ADAMS et al., 2010).

3.5. Adhesion of probiotic bacteria to intestinal cells in vitro

The assay testing the adhesion of probiotic bacteria to Caco-2 cells *in vitro* showed that adhesion was influenced significantly ($P \leq 0.05$) by the treatment (Figures 1 and 2). On the first day of analysis, the adhesion of *L. acidophilus* was higher ($P \leq 0.05$) in all treatments containing quinoa flour than in the control treatment. However, no difference was found ($P > 0.05$) for the adhesion of *B. animalis* subsp. *lactis* among treatments on the first day of storage. On day 28, the adhesion of *L. acidophilus* was higher ($P \leq 0.05$) in the treatment containing 2% quinoa flour than in the others. The adherence of *B. animalis* subsp. *lactis* on the last day of analysis was concentration-dependent: the higher the quinoa flour content, the greater the adhesion to Caco-2 cells ($P \leq 0.05$). The presence of quinoa flour in fermented milk may have favored the adhesion of probiotics, mainly of lactobacilli on day 1st of storage and bifidobacteria on day 28. The high amount of carbohydrates (74.3%), mainly starch, found in the flour may have helped the probiotic bacteria to adhere to Caco-2 cells. However, since the the differences found between control and supplemented treatments were below 1 log UFC/mL, they are not significant from the microbiological point of view. Compared to the initial number of cells (7-8 log CFU/mL), the proportion of each probiotic strain that adhered to the cell line used for the assay was relatively low for all fermented milk samples (3-5 log CFU/mL).

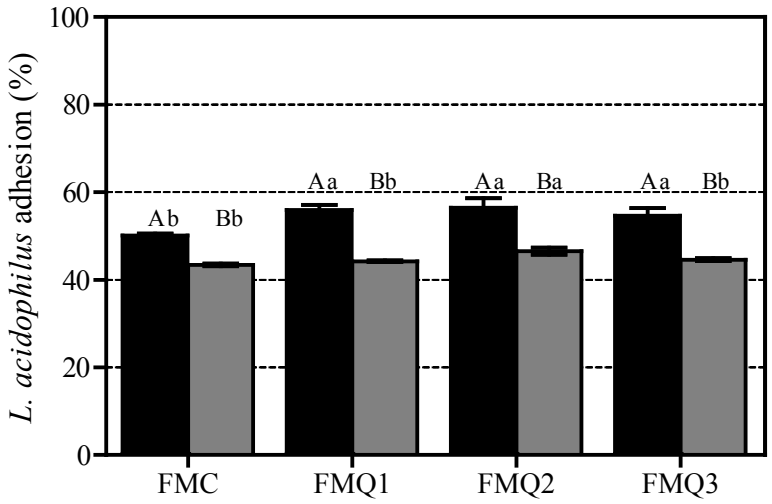

Figure 1. *L. acidophilus* adhesion (%) to Caco-2 cells, after 1 (■) and 28 (■) days of storage. FMC, fermented milk control, without quinoa flour; FMQ1, fermented milk supplemented with 1% quinoa flour; FMQ2, fermented milk supplemented with 2% quinoa flour and FMQ3, fermented milk supplemented with 3% quinoa flour. [a, b, c] Different lowercase letters denote significant differences ($P \leq 0.05$) among treatments for the same storage period. [A, B, C] Different capital letters denote significant differences ($P \leq 0.05$) for the same treatment during storage. n = 3.

The *in vitro* tests showed that besides being dependent on the matrix composition, the adhesion is also strain-specific. *L. acidophilus* showed lower rates (43.42-56.47%) of adherence to Caco-2 cells than bifidobacteria (53.23-65.89%) both at the beginning and end of storage of fermented milk. Certain probiotic strains may exhibit higher adhesion capacity due to specific physiological and biochemical characteristics, such as the presence of structures on the surface of bacterial cells that bind to intestinal mucus (Sanchez et al., 2008). Low adherence capacity to intestinal cells can result in reduced time to excretion of probiotics in feces. Saxelin et al. (2010) reported distinct excretion times for four different probiotic strains when consumed by healthy humans. These strains were administered in three types of matrices: yogurt, cheese and capsules. The longest excretion time was observed for *L. rhamnosus* GG, followed by *B. animalis* subsp. *lactis* BB-12, *P. freudenreichii* subsp. *shermanii* JS and *L. rhamnosus* LC705. Furthermore, the BB-12 and LC705 strains were excreted longer when consumed in the yogurt.

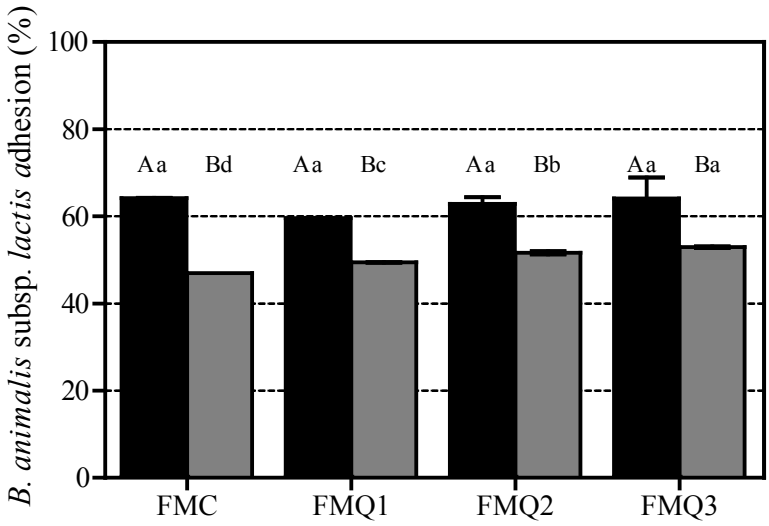

Figure 2. *B. animalis* subsp. *lactis* adhesion (%) to Caco-2 cells, after 1 (■) and 28 (■) days of storage. FMC, fermented milk control, without quinoa flour; FMQ1, fermented milk supplemented with 1% quinoa flour; FMQ2, fermented milk supplemented with 2% quinoa flour and FMQ3, fermented milk supplemented with 3% quinoa flour. [a, b, c] Different lowercase letters denote significant differences ($P \leq 0.05$) among treatments for the same storage period. [A, B, C] Different capital letters denote significant differences ($P \leq 0.05$) for the same treatment during storage. n = 3.

The ability of probiotic organisms to adhere is also influenced by the storage time of fermented milk, and a significant reduction ($P \leq 0.05$) was observed over time. This can be related with the low pH in the samples at the end of storage. Marcinakova et al. (2010) observed that pretreatment at pH 3.0 significantly reduced the adhesion of the *E. faecium* strains to IPEC-J2 cells. When evaluating the adhesion of *L. rhamnosus* GG to Caco-2 cells, Deepika, Rastall and Charalampopoulous (2011) also reported a reduction in bacterial adhesion during storage in food models simulating yogurt and ice cream, and they concluded that storage time affects bacterial adherence more than other factors such as fat and sugar contents. A reduction in the hydrophobicity of probiotic cells over time also happened and was linked to decreased adhesion to Caco-2 cells.

It is difficult to generalize the adherence of probiotic bacteria to Caco-2 cells *in vitro* to the situation of the human gastrointestinal tract because host defense systems, competition with the microbiota, mucosal desquamation and peristaltic flow can modify bacterial adhesion (Lebeer et al., 2008). However, *in vitro* assays are essential for understanding the mechanisms of adhesion and provide important information about the

differences among bacterial strains and species (Jensen et al., 2012) and among possible food formulations.

The ability to adhere to mucosal surfaces of the gut and the subsequent long- or short-term colonization has been one of the most common criteria for the selection of probiotic strains (Lebeer et al., 2008). To provide benefits, such as competitive exclusion of pathogenic from the intestinal epithelium or immunoregulation, in the large intestine, the probiotic must be capable of at least temporarily colonize the intestinal mucosa (Sanchez et al., 2008; Velez et al., 2007).

Several studies determined the adhesion of probiotics or potentially probiotic strains to several cell lines (Argyri et al., 2013; Ryu and Chang, 2013). However, few studies have been conducted to determine the effect of food or specific components on the adhesion properties of bacterial cells. The adhesion of probiotic organisms to intestinal cells may be influenced by the ingredients and by type of food matrix carrier of these microorganisms. Therefore, it is important to evaluate the interactions between food and bacterial surfaces because they can affect the physicochemical properties of bacterial cell surfaces and, consequently, their adherence to the intestinal mucosa (Deepika et al., 2011).

4. CONCLUSIONS

The present study showed that milk supplementation with 1-3% quinoa flour did not increase fermentation time. The viability of *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* Bb-12 in the fermented milk was satisfactory until the 28th day of refrigerated storage, ranging from 7 to 8 log CFU/mL for both microorganisms, and was not affected by the presence of quinoa flour. The bifidobacteria was more resistant to simulated GI conditions than the lactobacilli, but probiotic survival at the end of the *in vtiro* assay was not influenced by the quinoa flour. The additon of quinoa flour did not affect probiotic adhesion to Caco-2 cells as well, however, the *in vitro* adhesion to Caco-2 cells was reduced during storage.

ACKNOWLEDGMENTS

The authors acknowledge FAPESP for the financial support (Sao Paulo Research Foundation, grant 2009/12808-6).

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Capítulo 5

Este trabalho foi submetido ao *International Journal of Dairy Technology*

SURVIVAL OF PROBIOTICS IN THE PRESENCE OF COMMERCIAL DRUGS AND ITS RESISTANCE IN DIFFERENT MATRICES UNDER *IN VITRO* SIMULATED GASTROINTESTINAL CONDITIONS

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ABSTRACT

The effect of drugs on the survival of the strains *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* subsp. *animalis* BB-12 and matrix type on the resistance of probiotics under simulated gastrointestinal conditions were studied. La-5 and BB-12 were inhibited by 17 (28%) and 25 (41%) drugs, respectively. These drugs belonged to different groups, though they largely included antimetabolic, antihypertensive and analgesic drugs. Milk and inulin can protect probiotics during a simulated passage through the human gastrointestinal tract. These results suggest that is important to consider the co-administration of probiotic and drug therapy and that matrices play an important role in the functionality of probiotic fermented milk.

Keywords: Medicine, Gastrointestinal tract, Milk, Inulin, Lactic acid bacteria, Probiotics.

1. INTRODUCTION

According to the World Health Organization, probiotics are live micro-organisms that, when administrated in adequate amounts, offer health benefits to the host (FAO/WHO, 2002). Some of the beneficial effects of probiotic consumption include the improvement of intestinal health through the regulation of microbiota, as well as the stimulation and development of the immune system. These functional properties may vary due to various factors associated with the carrier food, including the ingredients used, manufacture processing, physicochemical properties, and storage conditions (Nagpal *et al.* 2012).

Several lactic acid bacteria (LAB) strains are well characterized and presently marketed as probiotics. The best-studied strains belong to the genera *Lactobacillus* and *Bifidobacterium*. In order to perform their beneficial effects, probiotic strains must survive the harsh conditions in the gastrointestinal tract (GIT): they must tolerate acid, bile and gastrointestinal enzymes, and then adhere to and colonize the intestinal epithelium (Vinderola *et al.* 2011).

Factors that are detrimental to the viability of probiotics in the stomach are low pH levels and the antimicrobial action of pepsin. The pH of the stomach generally ranges from 2.5 to 4.5, but it can be as low as pH 1 or pH 2 at higher rates of gastric juice secretion (Maragkoudakis *et al.* 2006). After passing through the stomach, ingested probiotics are faced with surviving in the small intestine, where they are exposed to pancreatin, bile salts, and a pH level of approximately 8.0. In addition to gastric resistance, the tolerance of probiotic bacteria to small intestine conditions may also be influenced by the diet of the host (Ranadheera *et al.* 2012).

In an effort to protect probiotics, a number of approaches for enhancing their survival in acid and enteric conditions have been investigated, including physical protection (microencapsulation) (Gebara *et al.* 2013), the use of authorized food ingredients (such as whey protein concentrate (WPC) and inulin) (Buriti *et al.* 2010), and food matrices (Ranadheera *et al.* 2012).

Inulin-type fructans may exert a protective effect as prebiotic food ingredients, thus improving the survival and activity of probiotic bacteria during the storage of probiotic foods, as well as the passage through the GIT. This ingredient increases resistance to pH changes and to the enzymes present in the GIT, which allows the

probiotic bacteria to reach the intestine at a higher viable cell concentration (Akalın *et al.* 2007; Buriti *et al.* 2010).

In addition to the harsh conditions of the GIT, the presence of non-antibiotic, orally administrated medications when individuals are under therapy may also cause a stress in the probiotic microorganisms (Todorov *et al.* 2008). It is well known that several anti-inflammatory drugs such as diclofenac potassium and other K^+ and Na^+ containing medications have negative effects on GIT microbiota after long-term application. However, medical doctors still prescribe these medications in combination with probiotic products. Therefore, individuals under drug therapy should be aware that these substances may reduce the beneficial effects of the probiotic bacteria. Despite the importance of this interaction, according to our knowledge, there are few papers dedicated to evaluating the effect of a wide spectrum of drugs on the growth of the commercial probiotic bacteria used in a variety of marketed products.

In light of the interest in protecting the probiotics during GIT transit, and also considering the possibility of negative interaction between probiotics and commercial drugs, the aims of this study were (1) to evaluate the effects of commercial drugs from different generic groups with special focus on that used in long-term treatment of chronic diseases on the growth of *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* subsp. *animalis* BB-12, and (2) to determine the effects of the matrix (MRS, milk and inulin) on the the survival of probiotic strains under simulated gastrointestinal conditions. The results may offer additional information to physicians, nutritionists, and pharmacists, and even to patients receiving drug therapy.

2. MATERIALS AND METHODS

2.1. Materials

Skim milk powder (SMP) (Nestlé, Araçatuba, São Paulo, Brazil), long-chain inulin (Rafitiline HP, Orafti, Oreye, Belgium, degree of polymerization of 23) and two commercial freeze-dried cultures (Chr. Hansen, Valinhos, Brazil), specifically the probiotics *L. acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12, were used in this study.

2.2. Effect of medications on the survival of probiotics strains

Seventy-one commercial medications (see Table 1) belonging to different groups (analgesic, anti-inflammatory, antihypertensive, etc) were purchased in a local drugstore. They were then solubilized in sterile water to achieve the concentrations indicated in Table 1. *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 were weighted and inoculated separately in 10% sterile reconstituted skim milk (RSM). They were then incubated at 37 °C for 18 h. MRS (Acumedia, USA) soft agar (0.8% agar) was inoculated with activated cultures of both types of probiotics strains (separately) in order to achieve a population of approximately 10^6 CFU/mL. After solidification of the agar, each medication (10 μL) was spotted onto the surface of the plates and incubated at 37 °C for 24 h. The plates were examined for the presence of inhibition zones around the spots of medication, and those presenting inhibition zones larger than 2 mm in diameter were further studied to determine the minimal inhibition concentration (MIC). Serial two-fold dilutions of the medications were prepared in sterile water, and 10 μL were spotted onto the surface of MRS soft agar plates that had been previously inoculated with *L. acidophilus* La-5 or *B. animalis* subsp. *lactis* BB-12 as it has been described before. The plates were incubated at 37 °C for 24 h and examined for the presence of inhibition zones around the spots. The MIC corresponded to the highest dilution that resulted in inhibition halos of at least 2 mm diameter (Todorov *et al.* 2008). The experiments were performed in duplicate.

2.3. Effect of matrices on survival of probiotic strains during *in vitro* simulated gastrointestinal conditions

The evaluation of probiotic survival in different matrices used in gastric and enteric simulated conditions test was performed according to the method described by Buriti *et al.* (2010), with modifications. According to the trial, *L. acidophilus* La-5 or *B. animalis* subsp. *lactis* BB-12 was activated as previously described. After the incubation period, 1 mL of the culture was inoculated into 9 mL of the selected matrices: MRS, RSM (reconstituted skim milk at 10% of solids) and RSM + inulin (RSMI). The inulin was added to reach a concentration of 1.5 g in the portion of food ready for consumption in order to promote the prebiotic effect. Aliquots of samples of each strain were diluted in 0.5% saline solution and transferred to 2 sterile flasks. There was

therefore a total of 12 flasks per strain (3 media x 2 gastrointestinal stages, in duplicate) that were subjected to *in vitro* conditions of the human gastrointestinal tract.

During the gastric phase, the pH was adjusted to 2.0 - 2.3 using sterile 0.5 N HCl. Sterile pepsin (3 g/L) (Sigma-Aldrich, St. Louis, MO, USA) and lipase (0.9 mg/L) (Amano lipase F-AP15, Aldrich Chemical Company, Milwaukee, WI, USA) solutions were added to samples. Flasks were incubated at 37 °C, with agitation of approximately 150 rpm. After 60 and 120 min, samples (1 mL) were withdrawn for the enumeration of viable cells. In the next step (the enteric phase), the pH was adjusted to 7.0 - 8.0 using a sterile alkaline solution containing bile (10 g/L) and pancreatin (1 g/L). This step was followed by incubation at 37 °C with agitation of approximately 150 rpm for 240 min. After 120 and 240 min after the beginning of the enteric phase, aliquots of 1 mL were withdrawn for the enumeration of viable cells. MRS medium was used for counting both microorganisms, and the plates of *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 were incubated at 37 °C for 48 h and 72 h, respectively. The experiment was performed in duplicate.

2.4. Statistical analysis

Results obtained from the *in vitro* test were analyzed by two-way ANOVA using STAT Software version 2.0. Mean values were compared using the Tukey test at $P < 0.05$. Different letters were used to label values with statistically significant differences among them.

3. RESULTS AND DISCUSSION

Many individuals who consume probiotic foods may be simultaneously undergoing drug treatment therapy of some kind. Therefore, it is important to determine the effect of drugs, with special attention to those used to treat chronic diseases and their effect on the survival of probiotic strains. The growth of *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 was inhibited by seventeen and twenty-five drugs, respectively (Table 1). These drugs belonged to different groups, including analgesic and anti-inflammatory non-steroidal drugs (NSAIDs) containing ibuprofen, diclofenac and ketoprofen, metamizole and paracetamol. Probiotics were affected mainly by medication containing metamizole among the analgesics tested. Some medications

containing 100 mg/mL of paracetamol did not inhibit the probiotic strains. However, *B. animalis* subsp. *lactis* BB-12 was inhibited by another medication with paracetamol at 60 mg/mL. This inhibition though was likely caused by the other components (carisoprodol or phenylbutazone) of the medication.

Most of the analgesic medications that were tested are commonly used by people of different ages, from infants to the elderly. Most of these medications are also freely commercialized and available without a prescription. Considering the negative effect of this group of medicines on the growth of probiotics, pharmacists and physicians can more accurately recommend which one can be used in a given case to avoid the loss of the therapeutic effect of probiotics.

The interference of drugs containing ibuprofen and diclofenac on the survival of probiotics has been reported by other authors (Todorov *et al.* 2011; Carvalho *et al.* 2009). Their inhibitory activity may be a consequence of the increase in the concentration of potassium ions in the gastric contents, which occurs as a result of the dissolution of diclofenac potassium in the stomach. The excess of potassium ions in the medium affects the viability of the bacterial cells (Carvalho *et al.* 2009). *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 were more affected by the anti-inflammatory drugs than the other drugs tested. These results are consistent with other studies (Todorov *et al.* 2008; Todorov *et al.* 2011; Botes *et al.* 2008).

Diclofenac potassium and ibuprofen inhibited the growth *of Lactococcus lactis* subsp. *lactis* HV219, a bacteriocin producer and potential probiotic strain (Todorov *et al.* 2007). Anti-inflammatory drugs (containing diclofenac potassium or sodium, ibuprofen), moderate diuretics (containing hydrochlorothiazide and triamterene), and neuroleptics (containing thioridazine hydrochloride) acted as growth inhibitors of potentially probiotic strains of *L. plantarum, L. rhamnosus, L. paracasei* and *L. pentosus*, isolated from a drink known as Boza (Todorov *et al.* 2008). Dimenhydrinate inhibited the growth of *L. rhamnosus* and *L. plantarum* (Todorov *et al.* 2008) but it did not affect the survival of *L. casei* Shirota and *L. casei* LC01 (Carvalho *et al.* 2009). In this study, neither probiotic strain evaluated was inhibited by dimenhydrinate.

Table 1. Effect of commercial drugs used in different therapies on the survival of *L. acidophilus* La-5 e *B. animalis* subsp. *lactis* BB-12, presented as diameter of inhibition zones in millimeters and minimal inhibitory concentration (MIC).

Drug (commercial name in Brazil)	Concentration (mg/mL)	Active substance	Drug class	*L. acidophilus* La-5	*B. animalis* subsp. *lactis* BB-12
				Inhibition (mm) [MIC (mg /mL)]	Inhibition (mm) [MIC (mg /mL)]
Aldactone	5	Spironolactone	Diuretic	0	12.00±0.00 [0.625]
Biprofenid	30	Ketoprofen	Analgesic, anti-inflammatory and antipyretic	15.00±0.00 [30]	14.50±0.71 [30]
Buscopan composto	2	Butylscopolamine	Antispasmodic and analgesic	15.00±0.00 [1; 25]	0
	50	Metamizole			
Cardilol	1.25	Carvedilol	Antihypertensive	0	13.50±2.12 [0.313]
Celebra	40	Celecoxib	Anti-inflammatory	0	14.00±0.00 [40]
Cloridrato de prometazina	5	Promethazine hydrochloride	Antihistamine	0	12.00±0.00 [1.25]
Cloridrato de propranolol	8	Propranolol hydrochloride	Antihypertensive	0	13.50±2.12 [8]
Cozaar	20	Losartan potassium	Antihypertensive (ACE inhibitor)	0	13.50±2.12 [2.5]
Diclofenaco potássico	10	Diclofenac potassium	Anti-inflammatory	10.00±0.00 [10]	17.00±0.00 [5]
Diovan Amlo Fix	32	Valsartan	Antihypertensive	0	17.50±3.54 [32; 1]
	1	Amlodipine besylate			
Dipirona sódica	100	Metamizole	Analgesic	15.00±0.00 [100]	12.00±0.00 [100]
Dorflex	60	Metamizole	Analgesic, anti-inflammatory and muscle relaxant	12.50±0.71 [30; 5; 3.5]	15.00±0.00 [15; 2.5; 1.75]
	10	Caffeine			
	7	Orphenadrine citrate			
Ebastel	2	Ebastine	Antihistamyne	0	15.00±0.00 [0.25]
Flamador	10	Ketoprofen	Analgesic, anti-inflammatory and antipyretic	0	11.50±0.71 [10]
Ibuprofene Biogaran	40	Ibuprofen	Anti-inflammatory	20.00±0.00 [20]	0
LipLess	20	Ciprofibrate	Hypolipidemic	11.00±0.00 [20]	12.00±0.00 [20]
Lisador	100	Metamizole	Analgesic, antispasmodic and antipyretic	13.50±2.12 [25; 0.5; 0.25]	15.00±0.00 [25; 0.5; 0.25]
	2	Adiphenine hydrochloride			
	1	Promethazine hydrochloride			

Loratadina	2	Loratadine	Antihistamine	27.50±3.54 [0.125]	16.00±1.41 [2]
Maleato de enalapril	2	Enalapril maleate	Antihypertensive	23.50 ± 2.12 [0.03]	17.50±3.54 [1]
	5	*Passiflora alata*			
Maracugina	2.5	*Erythrina mulungu*	Neurosedative	16.00±5.66 [2.5; 1.25; 1.25]	0
	2.5	*Crataegus oxyacantha*			
Meloxicam	3	Meloxicam	Anti-inflammatory	0	13.50±2.12 [0.75]
Metotrexato	0,5	Methotrexate sodium	Antimetabolic	35.00±7.07 [<0.01]	30.00±0.00 [<0.01]
	0.2	Dihydroergotamine mesylate			
Migraliv	20	Caffeine	Analgesic	20.00±0.00 [0.1; 10; 35]	0
	70	Metamizole			
	60	Paracetamol			
Mioflex	30	Carisoprodol	Analgesic and anti-inflammatory	0	15.00±0.00 [60; 30; 15]
	15	Phenylbutazone			
Motilium	2	Domperidone	Antiemetic	30.00±0.00 [0.125]	0
	60	Metamizole			
Neosaldina	6	Isometheptene mucate	Analgesic and antipyretic	13.00±1.41 [30; 3; 3]	15.00±0.00 [15; 1.5; 1.5]
	6	Caffeine			
Paracetamol	150	Paracetamol	Analgesic and antipyretic	16.00±1.41 [75]	0
Plasil	2	Metoclopramide hydrochloride	Antiemetic	0	12.00±0.00 [2]
Profenid enterico	20	Ketoprofen	Analgesic, anti-inflammatory and antipyretic	12.00±0.00 [20]	13.00±1.41 [20]
Rupafin	2.56	Rupatadine fumarate	Antihistamine	0	12.00±0.00 [0.64]

Following commercial drugs have no effect on the growth of *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12: AAS adulto (acetylsalicylic acid, analgesic and antipyretic, at 100 mg/mL) Ansiopax (*Piper methysticum,* anxiolytic, at 46.8mg/mL); Cezarette (desogestrel, contraceptive, at 15 mcg/mL), Clonotril (clonazepam, anxiolytic, at 0,1mg/mL); Cloxazolam (anxiolytic and sedative, at 0.4 mg/mL); Decongex plus (phenylephrine hydrochloride and brompheniramine maleate, decongestant of the upper respiratory tract**,** at 3 and 2.4 mg/mL); Diasec (loperamide hydrochloride, anti-diarrhoeal, at 0.4mg/mL); Dramin B6 (dimenhydrinate and pyridoxine hydrochloride, antiemetic, at 10 and 2 mg/mL); Ebastel (ebastine, antihistamynic, at 2 mg/mL); Cloridrato de fexofenadina (fexofenadine hydrochloride, antiallergic, at 36 mg/mL); Levoid (levothyroxine sodium, treatment of Thyroid problems, at 17.6 mcg/mL); Maxsulid (nimesulide beta-cyclodextrin, analgesyc, anti-inflammatory and antipyretic, at 80 mg/mL); Meticorten (prednisone, anti-inflammatory, at 4 mg/mL); Metiocolin B12 (DL-methionine and inositol, hepatoprotective, choline chloride and cobalamin at 20, 10, 5 mg/mL and 0.4 mcg/mL); Miosan (cyclobenzaprine hydrochloride, muscle relaxant, at 1 mg/mL); Omepramedi (omeprazole, proton pump inhibitor, at 4 mg/mL); Plaq (clopidogrel bisulfate, antihypertensive, at 15 mg/mL); Prelone (prednisolone, corticosteroid, at 1 mg/mL); Primosiston (ethinyl estradiol and norethisterone acetate, antihemorrhagic, at 0.4 and 0.002 mg/mL); Resfenol (paracetamol, chlorpheniramine maleate and phenylephrine hydrochloride, analgesyc and antipyretic, at 80 and 0.8 mg/mL); Selozok (metoprolol succinate, antihypertensive, at 10 mg/mL); Sinvalip (simvastatin, hypolipidemic, at 4 mg/mL); Somalgin cardio (acetylsalicylic acid, analgesic and antipyretic, at 20 mg/mL); Spasfon LYOC (phloroglucinol, antispasmodic, at 16 mg/mL); Tamisa 30 (gestodene and ethinyl estradiol, contraceptive, at 15 and 0.006 mcg/mL); Toragesic (kerotolac trometamol, analgesic, at 2 mg/mL); Transamin (tranexamic acid, antihemorrhagic, at 50 mg/mL); Tylenol (paracetamol and pseudoephedrine chloridrate, analgesic and antipyretic, at 100 mg/mL and 6 mg/mL); Tylex (paracetamol and codeine fosfate, analgesic and antipyretic, at 100 and 6 mg/mL); Vasativ (cilostazol, antiplatelet**,** at 20 mg/mL); Vertix (flunarizine dihydrochloride, calcium channel blocker, at 2 mg/mL).

Methotrexate sodium was found to have the lowest MIC (<0.01 mg) among all of the drugs evaluated. Methotrexate (2,4-diamino-N10-methyl propylglutamic acid, MTX) is one of the most widely studied and effective therapeutic agents available for the treatment of many solid tumors, hematologic malignancies, and autoimmune diseases. MTX has played a crucial role in the treatment of breast cancer, acute lymphatic leukemia (ALL), osteogenic sarcoma, choriocarcinoma, lung cancer, bladder carcinoma, brain medulloblastoma, primary central nervous system lymphoma, and chronic myeloid leukemia. Apart from its original use as a cancer chemotherapeutic agent, it is indicated for several other diseases such as psoriasis, multiple sclerosis, Crohn's disease, and rheumatoid arthritis (Abolmaali *et al.* 2013). Methotrexate acts specifically during the S phase of the cell division cycle, and its mechanism of action is related to the inhibition of folic acid metabolism which, in turn, is used in the synthesis of RNA and DNA precursors (Shen *et al.* 2012). It may have taken similar action on the strains used in this study, and may have therefore contributed to the greater inhibition observed.

The dosage of methotrexate sodium varies with the pathology that is being treated. A recent systematic review of the literature concerning the best dosage and route of methotrexate administration for arthritis rheumatoids showed that the optimal evidence-based dosing regimen is to start with 15 mg/week orally (0.2 mg/kg), to gradually escalate by 5 mg/month up to 25–30 mg/week or the highest tolerable dose (0.3 mg/kg) according to the patient's response and tolerance, and to subsequently switch to subcutaneous administration in the case of an insufficient response (Visser and Van der Heijde 2009). This dosage is well above its MIC obtained for each probiotic strain (<0.01 mg/mL). Therefore, the therapeutic action of probiotics may be completely suppressed in patients who use this drug.

The correct evaluation of possible drug interactions and probiotic bacteria depends on the MIC that is determined for these drugs. As shown in Table 1, the MIC for Biprofenid, an analgesic, anti-inflammatory and antipyretic, was 30 mg/mL for both strains. However, the daily dosage for this drug is 300 mg, and the MIC value associated with the volume of the human gastrointestinal tract indicates that the recommended daily dose will hardly affect the survival of probiotic bacteria.

However, it is important to consider the MIC when it comes to the drugs used to treat chronic diseases. *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 were inhibited by an antihypertensive containing carvedilol, propranolol hydrochloride,

valsartan, enalapril maleate or losartan, and a hypolipidemic drug containing ciprofibrate. These drugs can accumulate in the gastrointestinal tract after long-term use, and they affect the viability of probiotic cultures. Antihypertensive drugs are an even bigger problem in the case of co-administration with *B. animalis* subsp. *lactis* BB-12, since this probiotic was inhibited by all tested antihypertensives. Individuals undergoing lifelong therapy with drugs should be aware that these drugs may reduce the beneficial effects of probiotic bacteria. There is also a lack of studies about the interaction between probiotics and drugs used to treat specific diseases, such as cancer and chronic diseases (including diabetes, renal insufficiency, liver disorders and cardiovascular diseases).

On the other hand, the administration of probiotic strains along with antimicrobials can augment therapy efficacy. A study demonstrated that oral probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 treatment augmented the efficacious treatment of bacterial vaginosis with metronidazole. The antibiotics function by killing the pathogens, while *Lactobacillus* GR-1 and RC-14 are known to inhibit urogenital pathogen growth and adhesion, displace bacterial vaginosis organisms, downregulate vaginal inflammation and enhance immune defenses, all of which could explain the effectiveness of the combined treatment used in this study (Anukam *et al.* 2006).

To exert its beneficial effects on the host, probiotic bacteria should be viable in the product upon consumption and should also be able to reach the large intestine in amounts that are high enough to facilitate its colonization and proliferation (Shah 2000). Overall, there was a significant reduction ($P \leq 0.05$) in the population of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* La-5 during the simulation of the *in vitro* gastrointestinal tract conditions described herein. *B. animalis* subsp. *lactis* BB-12 were found to have higher rates of survival during the test compared to *L. acidophilus* La-5 in all tested matrices. Lower resistance was observed for *L. acidophilus* La-5 in the MRS medium. A good protection at the end of the *in vitro* test was observed when the probiotics were in the presence of milk and milk + inulin during the simulation, in the case of *L. acidophilus* La-5, or only in the presence of milk + inulin, in the case of *B. animalis* subsp. *lactis* BB-12 (Fig. 1). The survival rate of *B. animalis* subsp. *lactis* BB-12 in the MRS, RSM and RSMI treatments was 52%, 62% and 67%, respectively at the end of the *in vitro* test, while the survival of *L. acidophilus* La-5 was 17%, 29% and 44% in MRS, RSM and RSMI treatments, respectively.

There is new research in the literature that focuses on how the food matrix and dietary content interact with probiotic strains and how they protect these strains during gastrointestinal tract (Espírito-Santo *et al.* 2011). As reported by Martinez *et al.* (2011), food matrix composition affected the survival and growth of *L. amylovorus* during its passage through TIM-1, a dynamic system that simulates the human upper gastrointestinal tract.

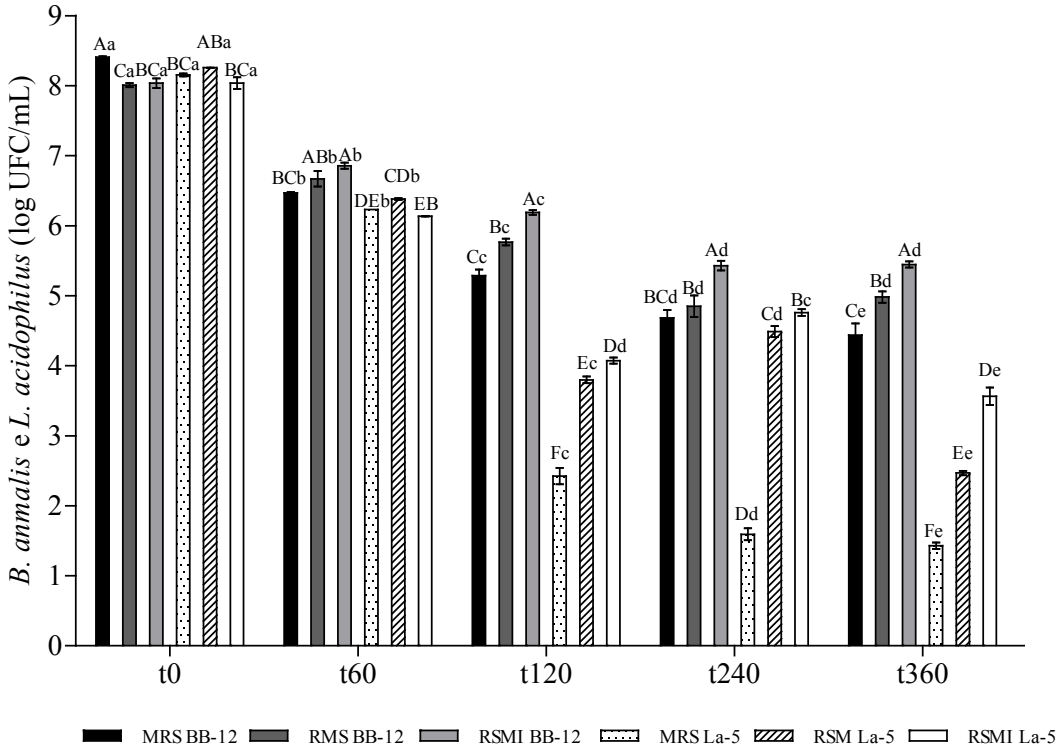

Figure 1. Survival of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* La-5 in different matrices during exposure to *in vitro* simulated gastrointestinal conditions for 360 min. [A, B, C] Different upper case letters denote significant differences ($P \leq 0.05$) among treatments for the same sampling period of the *in vitro* assay. [a, b, c] Different lower case letters denote significant differences ($P \leq 0.05$) among different sampling periods of the *in vitro* assay for the same trial. MRS BB-12: *B. animalis* subsp. *lactis* incorporated in MRS; RSM BB-12: *B. animalis* subsp. *lactis* incorporated in reconstituted skim milk; RSMI BB-12: *B. animalis* subsp. *lactis* incorporated in reconstituted skim milk with inulin; MRS La-5: *L. acidophilus* incorporated in MRS; RSM La-5: *L. acidophilus* incorporated in reconstituted skim milk; RSMI La-5: *L. acidophilus* incorporated in reconstituted skim milk with inulin. n = 3.

Oral and fecal recovery during and after administration of a combination of *Lactobacillus rhamnosus* GG and LC705, *Propionibacterium freudenreichii* subsp. *shermanii* JS, and *B. animalis* subsp. *lactis* Bb-12 as either capsules, yogurt, or cheese were examined. In the case of *P. freudenreichii* subsp. *shermanii* JS and *B. animalis* subsp. *lactis* BB-12, a matrix effect was found at the end of the intervention and in the recovery time during follow-up. Yogurt yielded the highest fecal quantity of JS and BB-12 strains. The administration matrix did not influence the fecal quantity of lactobacilli, but it did affect fecal counts of propionibacteria and bifidobacteria, which were lower when consumed in cheese (Saxelin *et al.* 2010).

About 2.5 L of gastric juice with a pH of approximately 2.0 are secreted daily by the human GIT. The pH of the stomach varies from 1.5 to 3.5, depending on the diet and on the intervals between meals. In addition, about 0.7 L of pancreatic juice are released into the small intestine daily, with a pH level of approximately 8 and 0.5% of salt. These substances represent chemical and enzymatic barriers to probiotic survival during food digestion and absorption (Charteris *et al.* 1998; Nag and Das 2013).

The buffer effect of milk proteins may have protected the cells against the deleterious effects of gastric juice. Thus, the food matrix and the conditions of the GIT may influence the survival of probiotics in the body. Individuals who ingest small quantities of food present low pH levels in the stomach (pH < 2.0), so the consumption of foods containing probiotic bacteria may result in the rapid destruction of these microorganisms, and the expected benefits would not occur. In these cases, probiotic bacteria must be ingested with foods containing components with buffering capacity, such as yogurt, milk, or other foods that are rich in proteins (Carvalho *et al.* 2009).

Milk can be fortified with other ingredients to improve the proliferation of probiotic bacteria. Prebiotics (particularly inulin) are usually used in milk fortification. Other authors have reported on the bifidogenic effect of inulin in dairy products. Oliveira *et al*. (2009) and Ozer *et al.* (2005) observed higher populations of bifidobacteria during storage in a food matrix to which inulin levels of 4.0 and 0.5%, respectively, had been added. Inulin can improve the viability of probiotics in a product. According to the results obtained in this study, inulin can also improve bifidobacteria survivability in the human gastrointestinal tract due to its ability to protect cells against damage caused by an acidic environment (Makras *et al.* 2005; Pimentel *et al.* 2012). In the food matrix, inulin binds to available water, producing a gel made up of a tridimensional network of microcrystals that interact and thus forming small aggregates

that occlude a large amount of water. This protection is probably associated with the resistance of inulin to hydrolysis caused by the GIT enzymes (Buriti *et al.* 2010; Franck 2008).

The effect of substitution of milk fat with inulin and whey protein concentrate (WPC) on *Lactobacillus acidophilus* La-5 resistance to simulated gastric and enteric conditions in symbiotic guava mousses under refrigeration or freezing were investigated by Buriti *et al*. (2010). The protective effect of inulin and WPC on *L. acidophilus* La-5 was shown to be more specific for the refrigerated products.

The probiotics *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 were incorporated into a fermented soy product containing inulin or okara or both. Inulin and okara failed to improve probiotic resistance during simulated GIT transit. However, the fermented soy product improved survival for both strains, particularly *B. animalis* subsp. *lactis* Bb-12, when compared to a freshly prepared culture in MRS under the same conditions, and so this matrix may be considered a good vehicle for the tested microorganisms. The authors reported that the degree of polimerization of inulin (which was found to be approximately 10) was responsible for the lack of inulin protection during the *in vitro* test (Bedani *et al.* 2013).

In the present study, the recovery of *B. animalis* subsp. *lactis* BB-12 viability after increasing pH up to 7.0, (i.e., during the enteric stage), did not occur, while the transfer from the acidic condition to the less damaging environment during the enteric phase, a slightly increased of the population of *L. acidophilus* La-5 was observed in the RSM and RSMI treatments. The reduced survival of tested strains was more intense during the gastric phase of the *in vitro* assay (Figure 2). Several studies have shown that probiotic cultures are strongly affected by exposure to simulated gastric fluids, depending on the strain, pH and time of exposure, but they were resistant to small bowel transit (Charteris *et al.* 1998, Kos *et al.* 2000). *B. animalis* subsp. *lactis* BB-12 was more resistant to acidic condition used in the *in vitro* test compared to *L. acidophilus* La-5 in all treatments (Figures 1 and 2), which is in agreement with other studies (Madureira *et al.* 2005; Guo *et al.* 2009).

4. CONCLUSIONS

A variety of commercial drugs inhibited the growth of probiotic strains. These results indicate that some medications cannot be used in combination with probiotic

products. Probiotic survival in the simulated GIT conditions was dependent upon the type of matrix. Inulin protected probiotics (especially *B. animalis* subsp. *lactis* BB-12) from the deleterious conditions of the gastrointestinal tract.

ACKNOWLEDGMENTS

The authors would like thank FAPESP (Sao Paulo Research Foundation, Sao Paulo, Brazil, grant 2009/12808-6) and CAPES (Ministry of Education, Brazilian Government, Brazil) for scholarships and financial support.

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Capítulo 6

CONCLUSÕES GERAIS

a. A qualidade do leite fermentado foi influenciada pelas diferentes composições das culturas láticas. A velocidade de acidificação foi menor nas amostras fermentadas por cultura pura de probióticos. Durante a fermentação, houve o consumo de lactose com concomitante produção de ácido lático, sendo que as bactérias que produziram a maior quantidade foram as homofermentativas *S. thermophilus* e *L. acidophilus.* As amostras dos tratamentos com maior teor de ácido acético ao final da fermentação foram aqueles contendo a bactéria *B. animalis* subsp. *lactis.* A razão molar determinada para os ácidos lático/acético para o tratamento Bb foi de 2,35:2, o que foi vantajoso do ponto de vista sensorial. Apenas *L. acidophilus* foi capaz de metabolizar o citrato, enquanto os níveis de piruvato aumentaram ligeiramente durante a fermentação. A sinérese reduziu ao longo do armazenamento e as populações dos micro-organismos probióticos atenderam o mínimo exigido pela legislação. As bifidobactérias apresentaram maior capacidade de sobrevivência e o tempo de armazenamento foi um fator determinante para a resistência das cepas ao ensaio *in vitro.* A avaliação sensorial mostrou que os leites fermentados pela cultura simples de *L. acidophilus* receberam as menores notas na avaliação sensorial, principalmente nos atributos sabor e aceitabilidade geral, com correlação negativa com a quantidade de ácido lático e correlação positiva com o teor ácido cítrico dos leites fermentados.

b. A adição de farinhas de frutas (banana, maça e uva) não alteraram a cinética de acidificação.Não houve diferença nos valores de acidez titulável entre os tratamentos ao final da estocagem, e os maiores valores de pH foram obtidos nos tratamentos com adição de farinhas aos 28 dias. A presença da farinha de banana aumentou as populações dos probióticos ao final da estocagem dos produtos, mas esse aumento não foi significativo do ponto de vista microbiológico. As farinhas de frutas protegeram o *L. acidophilus* durante a simulação das condições do TGI.

c. A adição de farinha de quinoa ao leite não influenciou o tempo de fermentação e resultou em produtos com maior pós-acidificação ao final do período de estocagem. A adição de farinha de quinoa não teve efeito positivo sobre a viabilidade dos micro-organismosdurante o armazenamento refrigerado dos leites fermentados. No entanto, foi observada tendência positiva da adição desse ingrediente na concentração de 3% sobre a

viabilidade de bifidobactéria após 28 dias de estocagem, uma vez que a população foi superior (0,87 log UFC/mL) em relação ao tratamento controle. Não houve efeito da adição da farinha de quinoa sobre a sobrevivência dos probióticos às condições simuladas do TGI. e sobre a capacidade de adesão dos probióticos às células Caco-2. No entanto, a adesão das cepas às células Caco-2 foi influenciada pelo tempo de estocagem dos produtos.

d. Houve efeito deletério de medicamentos na sobrevivência dos probióticos. Dentre os analgésicos as cepas probióticas foram inibidas principalmente pela dipirona sódica. O metotrexato apresentou a menor concentração mínima inibitória e alguns medicamentos usados no tratamento de doenças crônicas afetaram a sobrevivência dos probióticos. Este resultado é preocupante, visto que essas drogas podem acumular-se no intestino devido ao uso em longo prazo. A presença de leite ou inulina promoveu maior resistência dos probióticos no teste de sobrevivência às condições simuladas do TGI.

Anexos

Supplementary material 1. Parecer do Comitê de Ética em Pesquisa

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Câmpus de São José do Rio Preto

CEP Comitê de Ética em Pesquisa IBILCE-UNESP

PARECER CONSUBSTANCIADO
PROJETO DE PESQUISA

IDENTIFICAÇÃO

Nome do pesquisador: Sabrina Neves Casarotti
Departamento: Depto. Engenharia e Tecnologia de Alimentos
Instituição: IBILCE/UNESP
Projeto: "Efeito da associação de culturas láticas sobre a qualidade tecnológica de leites fermentados".

PARECER Nº 065/09

O projeto é de grande importância na adequação de cepas na produção de leites fermentados.

O projeto esta apresentado adequadamente com introdução, justificativa, material e métodos, casuística e bibliografia.

O objetivo está claro e a metodologia é adequada. Os riscos e/ou benefícios justificam a execução do projeto. Há infra-estrutura necessária e concordância da instituição em que será desenvolvida a pesquisa. Foi apresentado orçamento financeiro. Há declaração de que os resultados da pesquisa serão divulgados. Esta definido como será obtido o termo de consentimento e quem ficará responsável em obte-lo. O formulário TCLE apresentado contempla as informações sugeridas pelo CEP. As medidas para proteção dos indivíduos quanto ao monitoramento de coleta de dados pessoais são adequadas para garantir a segurança dos indivíduos e confidencialidade dos dados.

A pesquisadora atende os termos da resolução 196/96 do Conselho Nacional de Saúde, portanto o CEP aprova o projeto em questão.

(X) APROVADO
( ) COM PENDÊNCIA, máximo de 60 dias para atendimento
( ) RETIRADO
( ) NÃO APROVADO
( ) APROVADO, aguardar apreciação final da CONEP

Datas previstas para apresentação do relatório
1° relatório: outubro/2010 – **2° relatório:** outubro/2011 – **3° relatório:** outubro/2012

São José do Rio Preto, 07 de outubro de 2009.

Prof. Dr. Antonio José Manzato
Vice-Coordenador do CEP

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Supplementary 2. Modelo de ficha utilizada na análise sensorial.

Nome: _____Idade:_____ Nº amostra: _____

Você está recebendo uma amostra de leite fermentado. Por favor, prove-a e avalie cada item segundo a escala abaixo. Após experimentar cada amostra, tome um pouco de água e experimente a próxima.

9 - Gostei muitíssimo
8 - Gostei muito
7 - Gostei moderadamente
6 - Gostei ligeiramente
5 - Indiferente
4 - Desgostei ligeiramente
3 - Desgostei moderadamente
2 - Desgostei muito
1 - Desgostei muitíssimo

Aparência global: _____
Consistência: _____
Odor: _____
Sabor: _____
Aceitabilidade geral: _____