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DESENVOLVIMENTO DE IOGURTE PROBIÓTICO COM ADIÇÃO DE POLPA DE FRUTOS BRASILEIROS E FIBRA DIETÉTICA TOTAL

ANA PAULA DO ESPÍRITO SANTO

Tese apresentada para obtenção do grau de
DOUTOR

Orientadora: Prof ª. Drª. Maricê N. Oliveira

Co-Orientador: Prof. Dr. Attilio Converti

São Paulo

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Tese apresentada ao Programa de Pós-Graduação em Tecnologia Bioquimico-Farmaceutica da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, como requisito parcial para obtenção do título de Doutor em Tecnologia Bioquimico-Farmaceutica.

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Ana Paula do Espírito Santo

DEVELOPMENT OF PROBIOTIC YOGHURT WITH ADDITION OF BRAZILIAN`S FRUIT PULP AND TOTAL DIETETIC FIBER

Comissão Julgadora da
Tese para obtenção do grau de Doutor

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4º. examinador

São Paulo, 10 de fevereiro de 2012.

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RESUMO

ESPÍRITO SANTO, A.P.; CONVERTI, A.; OLIVEIRA, M.N. **Desenvolvimento de iogurte probiótico com adição de polpa de frutos brasileiros e fibra dietética total.** São Paulo. 2012. 112 p. Tese (Doutorado) – Faculdade de Engenharia Química e de Processo "G. B. Bonino", Universidade de Estudo de Gênova, Itália. Faculdade de Ciências Farmacêuticas, Universidade de São Paulo. Brasil.

Novas tendências para desenvolvimento de leites fermentados com elevado valor agregados são o uso de frutos da Amazônia e a utilização de subprodutos de certas frutas como forma de aproveitamento integral do fruto e para minimizar a produção de resíduos. Dentre os frutos da Amazônia o açaí (*Euterpe* oleracea Mart., Arecaceae) tem o maior potencial enquanto alguns subprodutos de frutos como as cascas de maçã, banana e maracujá são promissores como ingredientes especialmente devido ao seu conteúdo em fibras dietéticas solúveis prebióticas como pectina e frutooligossacarídeos, que conferem propriedades funcionais além das características nutricionais das frutas. Assim, esse trabalho visou o desenvolvimento de iogurte probiótico com adição de polpa de frutos brasileiros e fibra dietética total. Os efeitos da suplementação do leite com polpa de açaí e fibras de maçã, banana e maracujá e, diferentes bactérias probióticas - *Lactobacillus acidophilus* L10, *Bifidobacterium animalis ssp. lactis* Bl04 e B94 e *Bifidobacterium longum* Bl05 na cinética de acidificação, viabilidade dos probióticos, perfil de ácidos graxos, textura, reologia e microestrutura foram estudados. A polpa de açaí favoreceu uma maior contagem de *L. acidophilus* L10, *B. animalis ssp. lactis* Bl04 e *B. longum* Bl05 em relação aos respectivos controles ao final de quatro semanas de vida de prateleira. Além disso, em relação aos controles sem polpa, a polpa de açaí aumentou o conteúdo de ácidos graxos mono e poliinsaturados e a produção de ácido α-linolênico (ALA) e ácido linoléico conjugado (CLA) em iogurtes desnatados co-fermentados com *B. animalis ssp. lactis* cepas Bl04 e B94. Todas as fibras foram capazes de aumentar a concentração de ácidos graxos de cadeia curta e poliinsaturados nos iogurtes, mas, apenas as fibras de maçã e banana aumentaram a viabilidade das bactérias probióticas durante a vida de prateleira em relação aos controles sem fibra. Foi observado um efeito sinérgico entre o tipo de fibra e a cepa probiótica sobre o teor de CLA. Por outro lado, a quantidade de ALA foi significativamente aumentada pela adição de fibra de banana, independentemente da cepa probiótica utilizada. A fibra de maracujá promoveu o aumento CLA em todos os iogurtes probióticos. Os resultados demonstram, pela primeira vez, que tanto a polpa de açaí quanto as fibras oriundas do subproduto do processamento de maçã, banana ou maracujá podem melhorar o perfil de ácidos graxos e a viabilidade de bactérias probióticas. Além disso, a fibra de casca de maracujá teve um efeito positivo sobre a textura de iogurtes desnatados co-fermentados por bifidobacteria.

Palavras-chave: Iogurte; Probiótico; Textura; Viabilidade; Ácido linoléico conjugado.

RIASSUNTO

ESPÍRITO SANTO, A.P.; CONVERTI, A.; OLIVEIRA, M.N. **Sviluppo del iogurt probiotici con aggiunta di polpa di frutta brasiliana e fibra dietetici totale.** 2012. 112 p. Tese (Dottorato di ricerca) – Facoltà di Ingegneria Chimica e di Processo "G. B. Bonino", Università degli Studi di Genova, Itália. Facoltà di Scienze Farmaceutiche, Università di São Paulo, São Paulo, Brasile.

Nuove tendenze per lo sviluppo di prodotti fermentato di latte con alto valore aggiunto sono l'uso dei frutti delle Amazzoni e l'utilizzo di alcuni sottoprodotti della frutta come un modo per sfruttare al massimo il frutto e per minimizzare la produzione di rifiuti. Tra i frutti dell'Amazzonia l´açai (*Euterpe oleracea* Mart., Arecaceae) ha il maggior potenziale e come sottoprodotti di alcuni frutti la mela, banana e frutto della passione come ingredienti sono particolarmente promettenti per il suo contenuto di fibra alimentare, come prebiotico - pectina e fruttooligosaccaridi solubile, che conferiscono proprietà funzionali in aggiunta alle caratteristiche nutrizionali della frutta. Quindi, questo studio a come scopo lo sviluppo di yogurt probiotico con polpa di frutta dal Brasile e fibra alimentare totale. Gli effetti della supplementazione di latte con fibra di polpa di açai e frutta mela, banana e frutto della passione, e differenti batteri probiotici - *Lactobacillus acidophilus* L10, *Bifidobacterium animalis* ssp. Bl04 *lactis* e *Bifidobacterium longum* Bl05, B94 en la cinetica di acidificazione, viabilità dei probiotici, profilo degli acidi grassi, consistenza, reologia e microstruttura sono stati studiati. La polpa di açaí ha aumentato la conta di *L. acidophilus* L10, *B. animalis* ssp. *lactis* Bl04 e *B. longum* Bl05 dopo 28 giorni di conservazione. Inoltre, l'aggiunta di polpa di açaí ha aumentato il contenuto di acidi grassi mono e polinsaturi negli yogurt probiotici e la produzione degli acidi α-linolenico (ALA) e linoleico coniugato (CLA) in yogurt co-fermentati con *B. animalis* ssp. *lactis,* ceppi Bl04 e B94. Le fibre di mela e banana hanno aumentato la vitalità probiotica durante la shelf-life, mentre tutte le fibre sono state in grado di aumentare il contenuto in acidi grassi a catena corta e acidi grassi polinsaturi degli yogurt rispetto ai corrispondenti controlli senza fibra. È stato osservato un effetto sinergico tra il tipo di fibra ed il ceppo probiotico sul contenuto di CLA, e la quantità di ALA è risultata maggiore negli yogurt fermentati con fibra di banana. La fibra del frutto della passione ha promosso un incremento significativo nel contenuto di CLA in tutti gli yogurt in rispetto ai controlli. I risultati dimostrano, per la prima volta, che le fibre di frutta sono in grado di migliorare il profilo di acidi grassi di yogurt probiotici e sottolineano l'opportunità di utilizzare le fibre dei sottoprodotti della lavorazione di frutta per sviluppare nuovi latti fermentati ad alto valore aggiunto. La fibra del frutto della passione ha ridotto significativamente il tempo di fermentazione in tutti gli yogurt scremati, ad eccezione di quello co-fermentato da *B. lactis* Bl04, e promosso un aumento significativo della conta di *B. lactis* Bl04 nello yogurt. In generale, il tipo di latte ha influenzato la conta dei probiotici più che la presenza di fibra. Dopo quattro settimane di conservazione a freddo, la fermezza, la consistenza (ad eccezione del ceppo NCFM di *L. acidophilus*) e la coesione sono state superiori in tutti gli yogurt scremati contenenti fibra del frutto della passione. I risultati dimostrano, per la prima volta in questo campo, che l'impiego di açaí o di sottoprodotti della lavorazione di mela, banana o frutto della passione nella formulazione di yogurt probiotici può migliorare il profilo di acidi grassi e la vitalità di batteri probiotici. Inoltre, la fibra del frutto della passione ha contribuito a migliorare i parametri di testura degli yogurt scremati co-fermentati da bifidobatteri.

Parole-chiave: Yogurt; Probiotici; Textura; Viabilità; Acido linoleico coniugato.

ABSTRACT

ESPÍRITO SANTO, A.P.; CONVERTI, A.; OLIVEIRA, M.N. **Development of probiotic yoghurt with addition of Brazilian´s fruit pulp and total dietary fiber.** 2012. 112 p. PhD Thesis – Chemical Engineering and Process Faculty "G. B. Bonino", University of Genoa, Italy. Pharmaceutial Sciences Faculty, São Paulo University, Brazil.

New trends for development of fermented milk products with high added value are the use of fruits from Amazon, and the use of by-products of certain fruits as ingredients as a way to take full advantage of the fruit and to minimize the waste. Among the fruits of the Amazon, açai (*Euterpe oleracea* Mart., Arecaceae) has the most potential, and byproducts of some fruits such as apple peels, banana and passion fruit as ingredients are especially promising because of its content in dietary fiber such as pectin and soluble prebiotic fructooligosaccharides, which confer functional properties in addition to the nutritional characteristics of fruits. Thus, this study aimed the development of probiotic yoghurt with added fruit pulp from Brazil and total dietary fiber. The effects of supplementation of milk with acai pulp fiber and apple, banana and passion fruit, and different probiotic bacteria - *Lactobacillus acidophilus* L10, *Bifidobacterium animalis* ssp. Bl04 *lactis* and *Bifidobacterium longum* B94 and Bl05 on the kinetics of acidification and viability of probiotics, fatty acid profile, texture, rheology and microstructure were studied. The açaí favored a higher count of *L. acidophilus* L10, *B. animalis* ssp. and *B. lactis* Bl04 Bl05 *longum* compared with their controls after four weeks of shelf life. Moreover, compared to controls without pulp, the pulp of acai increased the content of mono and polyunsaturated fatty acids and the production of α-linolenic acid (ALA) and conjugated linoleic acid (CLA) in nonfat yogurt co-fermented with B. *animalis* ssp. *lactis* strains Bl04 and B94. All fibers were able to increase the concentration of short chain fatty acids and polyunsaturated fats in yogurt, but only the apple and banana fibers increased the viability of probiotic bacteria during shelf life compared to controls without fiber. We observed a synergistic effect between the type of fiber and probiotic on the CLA content. On the other hand, the amount of ALA was significantly increased by the addition of banana fiber, regardless of the probiotic strain used. The passion fruit fiber promoted an increase CLA in all probiotic yoghurts. The results point out the applicability of adding whether açai or apple, banana or passion fruit by-products in the formulation of probiotic yoghurts to improve the fatty acids profile and to uphold the desirable probiotic counts during four weeks of cold storage. In addition, the passion fruit fiber helped to enhance the texture parameters in skim yoghurts co-fermented by bifidobacteria.

Key-words: Yoghurt; Probiotic; Texture; Viability; Conjugated linoleic acid.

1. INTRODUÇÃO

O consumo de alimentos regionais é recomendado pela Organização para Alimentos e Agricultura das Nações Unidas (FAO, 2008) para o desenvolvimento sustentável das comunidades agrícolas indígenas e não indígenas em áreas de alta diversidade biológica. Entre as diretrizes da Política Nacional de Alimentação e Nutrição (Portaria nº 710 de 10/06/99) consta o resgate da cultura alimentar regional de populações indígenas e não indígenas, através de ações que levem ao aumento do consumo, desenvolvimento de técnicas de processamento e comercialização de alimentos vegetais nativos. Estas ações devem passar, necessariamente, pelo crivo da segurança alimentar, que visa não apenas a quantidade, mas também, a qualidade do alimento consumido. Muitas das frutas nativas brasileiras, como o açaí (*Euterpe olaeracea*, Arecaceae) permanecem como curiosidades alimentares, apesar Kuskoski *et al.* (2005) sugerirem o aumento do consumo mundial de frutas tropicais. Um bom incentivo para a necessária integração entre os interesses sócio-econômicos e ambientais que envolvem o alimento regional é avaliar as propriedades nutricionais e funcionais dos frutos nativos e desenvolver novos produtos alimentícios com estas frutas.

A polpa de açaí tem sido considerada uma super-fruta devido à sua atividade anti-oxidante demonstrada *in vitro* e *in vivo*, principalmente devido ao seu conteúdo de compostos fenólicos, tais como a sua antocianinas (LICHTENTHALER *et al*, 2005). Além disso, o extrato bruto de açaí causou a vasodilatação e conseqüente redução da pressão arterial em ratos hipertensos, provavelmente com a participação de compostos fenólicos (ROCHA *et al*, 2007). Pesquisas mostram que a ingestão diária de frutas e vegetais ricos em compostos antioxidantes é de grande valor para prevenir o estresse oxidativo (STEINBERG, 1995). Há evidências significativas de que o consumo regular de alimentos ricos em flavonóides, reduz o risco de morte por infarto agudo do miocárdio (HERTOG *et al*, 1993; KNEKT, 1996), e a incidência de acidente vascular cerebral em idosos (KELI *et al*, 1996).

Segundo o Ministério da Agricultura do Brasil (BRASIL, 2008), a produção anual de frutos tem sido de mais de 38 milhões de toneladas, gerando cerca de 471,8 milhões dólar através da exportação de frutas frescas em 2006, o que representa um aumento de 95% em relação a 2002. O crescimento da produção de frutos tem permanecido na ordem dos 50% ao ano (BRASIL, 2008). Por outro lado, o desperdício de alimentos, especialmente frutas, é uma realidade em todo o mundo (FAO, 2008). No processamento de frutas para produção de polpa, em torno de 65-70% do peso do fruto é desprezado (OLIVEIRA *et al*., 2002), o que contribui para o agravamento do impacto ambiental gerado pelo lixo orgânico (LOUSADA Jr. *et al*., 2006). A tendência que começou nos

últimos anos 70, de usar subprodutos de certas frutas como ingredientes para novos alimentos, pode ajudar a minimizar este desperdício (OLIVEIRA *et al*., 2002). Entre os subprodutos de frutos promissores como ingrediente para a indústria de alimentos estão as cascas de maracujá, maçã e banana, especialmente por causa do seu conteúdo em fibras dietéticas solúveis como pectina e frutooligossacarídeo, que confere propriedades funcionais, além de aspectos nutricionais da fruta (STAFFOLO *et al.* 2004).

A crescente demanda por alimentos funcionais tem impulsionado os investimentos na área de pesquisa e desenvolvimento pelas indústrias de alimentos (MICHIDA *et al*, 2006). No Brasil, o Ministério da Saúde, através da Agência Nacional de Vigilância Sanitária (ANVISA), regulamentou os Alimentos Funcionais através das resoluções: ANVISA nº 18/99 e 19/99, que preconizam que o alimento ou ingrediente que alegar propriedades funcionais ou de saúde pode produzir efeitos metabólicos e ou fisiológicos e ou manutenção geral da saúde no que diz respeito ao papel fisiológico dos nutrientes e não nutrientes e à redução de risco de doenças, não sendo permitidas alegações de saúde que façam referência à cura ou prevenção de doenças (BRASIL, 1999a; BRASIL, 1999b).

De acordo com Food Processing (2009) e Granato *et al* (2010), o mercado de alimentos funcionais no Brasil foi responsável por 1% (U$ 500 milhões) das vendas totais de alimentos em 2007 sendo a maior parte deste mercado (65 %) composta por produtos probióticos. Mundialmente, os ingredientes, suplementos e alimentos probióticos compõe um mercado de U$ 16 bilhões em 2008 e estima-se que o vende destes produtos pode chegar a 19,6 bilhões dólares em 2013 (GRANATO *et al*, 2010). O mercado de alimentos funcionais, tais como probióticos tem experimentado um crescimento constante de 5% ao ano (STANTON *et al*, 2005; GRANATO *et al*, 2010). Entre as diversas espécies de probióticos, as do gênero *Lactobacillus* foi responsável por 61,9% das vendas totais em 2007 (FOOD PROCESSING, 2009).

Na fabricação de produtos lácteos fermentados são empregadas bactérias ácido-láticas dos gêneros Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Bifidobacterium, Propionibacterium, que fermentam açúcares, produzindo ácido lático como principal produto da fermentação, o que acidifica o leite impedindo o desenvolvimento de bactérias nocivas à qualidade do produto final e à saúde humana (FARIA *et al*, 2006). Os diferentes leites fermentados apresentam fases de produção similares, como padronização do conteúdo de gordura, aumento dos sólidos não gordurosos do leite, homogeneização, desaeração e tratamento térmico. No entanto, alguns aspectos tecnológicos precisam ser observados para a manutenção da viabilidade das culturas probióticas como a determinação do tipo de cultura inicializadora mais adequada, temperatura, tempo de incubação e taxa de inoculação (FARIA *et al*, 2006). Uma das culturas

inicializadoras mais utilizadas é uma mistura simbiótica de *Streptococcus thermophilus* e *Lactobacillus delbrueckii* subsp. *bulgaricus*. O *S. thermophilus* cresce rapidamente, produzindo ácido lático e dióxido de carbono a partir da lactose do leite, o que estimula o crescimento de *L. bulgaricus* (OLIVEIRA & DAMIN, 2003). Por outro lado, a atividade proteolítica do *L. bulgaricus* gera peptídeos e aminoácidos que foram utilizados para a manutenção do *S. thermophilus* na cultura (OLIVEIRA & DAMIN, 2003). Enquanto o *Streptococcus* é responsável pela redução do pH até, aproximadamente, 5, os *Lactobacilus* reduzem o pH até 4. Durante o processo fermentativo ocorrem mudanças na textura, aroma e sabor do leite, pela presença de ácido lático, acetaldeído, ácido acético e diacetil, que inibem a proliferação de microrganismos patogênicos (SAXELIN *et al*, 1999; REID *et al*, 2003).

Nos últimos anos, tem havido um aumento de produtos lácteos fermentados com adição de suco de frutas ou flavorizantes para melhorar a palatabilidade destes alimentos, no entanto, as frutas empregadas para este fim, quase sempre, são as adaptadas a climas temperados como maçã, morango ou pêssego (BRANDÃO, 2002). Do ponto de vista tecnológico, a adição de polpa de fruta nativa ao leite fermentado pode representar problemas ou benefícios tecnológicos, especialmente no que se refere à viabilidade dos probióticos durante a vida de prateleira do produto. Por outro lado, a presença da polpa de fruta pode incrementar os aspectos nutricionais e funcionais do alimento, especialmente no que se refere ao potencial antioxidante e ao teor de fibras prebióticas.

O desenvolvimento de produtos lácteos adicionados com frutas nativas brasileiras pode contribuir para o desenvolvimento sustentável das comunidades que habitam áreas de grande diversidade de espécies frutíferas o que, segundo Homma (2005), constitui uma excelente maneira de combater a biopirataria pois transforma os recursos da biodiversidade em atividades econômicas que geram emprego e renda para a população. Por outro lado, o uso de subprodutos do processamento de frutas como ingrediente para formulação de novos produtos alimentares com alta aceitação no mercado, como os produtos lácteos probióticos, pode ajudar a minimizar o impacto ambiental gerado pelo descarte de refugo da indústria de alimentos, além de contribuir para aumentar a ingestão de fibra dietética.

Com base nessas evidências, o principal objetivo desta tese foi o desenvolvimento de iogurte probiótico com adição de polpa de fruta brasileira e de fibra dietética total obtida dos subprodutos do processo de fabricação de polpa.

Os objetivos específicos foram:

1. Formulação de iogurtes co-fermentados por diferentes cepas de bactérias probióticas com polpa de açaí.

a. Avaliar o efeito da adição de polpa de açaí sobre a viabilidade das bactérias inicializadoras e probióticas em iogurtes durante quatro semanas de armazenamento a frio;
b. Avaliar o efeito da polpa de açaí sobre o tempo de fermentação e sobre os parâmetros físico-químicos, como pH, acidez total titulável e teor de lactose;
c. Determinar a influência da adição de polpa de açaí, uma fruta rica lipídios, nos perfis de ácidos graxos dos iogurtes formulados, especialmente no que diz respeito à produção de CLA.

2. Formulação de iogurtes co-fermentados por quatro diferentes cepas de bactérias probióticas adicionados de fibras dietéticas totais obtidas dos subprodutos do processamento de: maçã, banana ou maracujá.
a. Verificar o possível efeito prebiótico das fibras dietéticas totais sobre as cepas probióticas testadas;
b. Analisar do pós-acidificação e acidez titulável total;
c. Avaliar as mudanças no perfil de ácidos graxos dos iogurtes promovido pela combinação de fibras de frutas e diferentes cepas probióticas.

3. Formulação de iogurtes integrais e desnatados co-fermentados por quatro diferentes cepas de bactérias probióticas com fibra dietética total de cascas de maracujá, comparar o efeito do tipo de leite e da adição de fibra de maracujá sobre:
a. Parâmetros cinéticos;
b. Pós-acidificação;
c. Contagem de bactérias inicializadoras e probióticas durante quatro semanas de armazenamento a frio;
d. Parâmetros de textura.

2. INTRODUCTION

The recovery of regional foods is recommended by Food and Agriculture Organization of the United Nations (FAO, 2008) for the sustainable development of indigenous and non-indigenous farming communities in areas of high biological diversity. The enhancement of regional food culture of, through actions that lead to the increase consumption of products based on native plants is among the directives of the Brazilian National Policy on Food and Nutrition (Order nº 710, 10/06/99); (BRASIL, 2003). These actions must pass, necessarily, by the sieve of food safety, which aims not only the quantity but also the quality of the food consumed.

Many of the Brazilian native fruits such as açaí (*Euterpe olaeracea*, Arecaceae) remain as food curiosities, despite Kuskoski *et al*. (2005) have suggested the increase in the world consumption of tropical fruits. A good incentive for the necessary integration between socio-economic and environmental interests involving the regional food is to assess the nutritional and functional properties of native fruits and development of new food products with these fruits.

The açaí pulp has been considered a super-fruit due to the considerable anti-oxidant activity of its phenolic compounds such as anthocyanins (LICHTENTHALER *et al*, 2005). In addition, the crude extract of açaí caused the vasodilation and consequent reduction in blood pressure in hypertensive rats, probably with the participation of phenolic compounds (ROCHA *et al,* 2007). Some surveys show that the intake of fruits and vegetables rich in antioxidant compounds is of great value to prevent oxidative stress (STEINBERG, 1995). There is significant evidence that regular consumption of foods rich in flavonoids reduces the risk of death from acute myocardial infarction (HERTOG *et al*., 1993; KNEKT, 1996), and the incidence of stroke in elderly (KELI *et al,* 1996).

According to the Brazilian Ministry of Agriculture (BRASIL, 2008), the annual fruit production has being over 38 million tons, generating US$ 471.8 million through exportation of fresh fruits in 2006, representing an increase of 95% compared to 2002. The growth of fruit production has remained the order of 50% per annum (BRASIL, 2008). Moreover, the waste of food, especially fruit, is a worldwide reality (FAO, 2008). In the processing of fruit for pulp production, around 65-70% of the fruit weight is despised (OLIVEIRA *et al*., 2002), which contributes to worsening environmental impact generated by organic waste (LOUSADA Jr. *et al*., 2006). The trend, which began in the last 70's, to use by-products of certain fruits as ingredient for new foods, can help minimize this waste (OLIVEIRA *et al*., 2002). Among the promising fruits by-products as ingredient for food industry are the peels of passion fruit, citrus and banana, especially because of its content in soluble dietary fibers such as pectin and fructooligosaccharide, which confers functional properties in addition to nutritional aspects of fruit (STAFFOLO *et al*. 2004).

The growing demand for functional foods has driven investments in the area of research and development by the food industries (MICHIDA *et al*, 2006). In Brazil, the Ministry of Health through the National Health Surveillance Agency (ANVISA), requires that the food or ingredient that claim functional properties is not allowed to make reference to the cure or prevention of disease (BRASIL, 1999a; BRASIL, 1999b). For those nutrients with functions fully recognized by the scientific community, will not need the demonstration of effectiveness or analysis of the same claim for functional in labelling (ANVISA, 3.3 of Resolution No. 18, 2009).

According to Food Processing (2009) *in* Granato et al (2010), the market of functional foods in Brazil was responsible for 1% (US$ 500 thousand) of the total food sales in 2007 and, the major part of this market (65%) is composed by probiotic products. Globally, the probiotic ingredients, supplements, and foods make a market of US$16 billion in 2008 and is estimated that the sells of these products can reach US$19.6 billion in 2013 (GRANATO et al, 2010). The market for functional foods such as probiotics has experienced a steady growth of 5% per year (STANTON *et al*, 2005; GRANATO *et al*, 2010). Amongst the diverse species of probiotics, the *Lactobacillus* genus is responsible for 61.9% of total sales in 2007 (FOOD PROCESSING 2009).

In the production of fermented milk are employed some genera of acid-lactic bacteria such as *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Bifidobacterium* and *Propionibacterium*, which can ferment sugars preventing the development of harmful microorganisms to human health and to the quality of final product (FARIA *et al,* 2006). The various fermented milk products have similar stages of production, such as fat content standardization, increase in non-fat dry milk, homogenization, de-aeration and heat treatment. Peculiarities of the technological process of production of fermented milks concern mainly to the rate of inoculation, temperature of fermentation, final pH and, in the production of probiotic products, the selection of the probiotic cultures (FARIA *et al*, 2006).

One of the most used starter cultures is a symbiotic mix of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The *S. thermophilus* grows rapidly, producing lactic acid and carbon dioxide from the lactose in milk, which stimulates the growth of *L. bulgaricus*. Moreover, the proteolytic activity of *L. bulgaricus* generates peptides and amino acids to be used for the maintenance of *S. thermophilus* in culture (OLIVEIRA & DAMIN, 2003). While *Streptococcus thermophilus* is responsible for reducing the pH to approximately 5, the *Lactobacillus bulgaricus* reduces the pH until 4. During the fermentation process, occur changes in texture, aroma and flavor of milk because of the presence of lactic acid, acetaldehyde, acetic acid and diacetil, which inhibit the proliferation of pathogenic microorganisms (SAXELIN *et al*, 1999; REID *et al*, 2003).

In recent years, there has been an increase in new fermented milk products with addition of fruit juice or flavoring agents to improve the palatability of food. Nevertheless, the fruit used for this purpose, such as apple, strawberry or peach are adapted to temperate climates (BRANDÃO, 2002). From the technological point of view, the addition of native fruit pulp to the fermented milk can provide technological benefits or problems, especially regarding the viability of probiotics during the shelf life of the product. Moreover, the presence of the fruit pulp can increase the functional aspect of the product, especially the antioxidant potential and prebiotics fiber content.

The development of dairy products added with Brazilian native fruit may contribute for sustainable development of communities that inhabit areas of great diversity of plant species because, according Homma (2005), the best way to fight biopiracy is to transform the resources of biodiversity in economic activities that generate employment and income for the population. Similarly, the use of by-products from the processing of fruits as ingredient new food products with high market acceptance, as the probiotics dairy products, can help minimize the environmental impact and increase the intake of dietary fiber.

Based on these evidences, the major aim of this Thesis was the development of probiotic yoghurt with addition of Brazilian´s fruit pulp and total dietary fiber.

The specific objectives were:

1. Formulation of yoghurts co-fermented by different strains of probiotic bacteria with açaí pulp.

a. Evaluate the effect of açai pulp addition on the viability of starter and probiotic bacteria in the formulated yogurts during four weeks of cold storage;

b. Asses the effect of açai pulp on fermentation time and physical-chemical parameters such as pH, titratable acidity and lactose content;

c. Determine the influence of the addition of açaí pulp, a lipids rich fruit, on the fatty acid profiles of the yoghurts, especially on the CLA production.

2. Formulation of yoghurts co-fermented by four different strains of probiotic bacteria with total dietetic fiber from apple, banana and passion fruit processing by-products:

a. Verify the possible prebiotic effect of the fruit total dietary fibers on the probiotic strains tested;

b. Analyse of the post-acidification and total titratable acidity;

c. Evaluate the changes on the fatty acids profile of the yoghurts promoted by the combination of fruit fibers and different probiotic strains.

3. Formulation of plain and skim yoghurts co-fermented by four different strains of probiotic bacteria with total dietetic fiber from passion fruit rinds, compare the effect of the milk type and of the passion fruit fiber on:

a. Kinetic parameters;

b. Post-acidification;

c. Counts of starter and probiotic bacteria during four weeks of cold storage;

d. Texture parameters

CHAPTER I

INFLUENCE OF FOOD MATRICES ON PROBIOTIC VIABILITY – A REVIEW FOCUSING ON THE FRUITY BASES

1.1. Introduction

Gut health is a common target in the development and consumption of functional foods, and some market figures point out this reality, showing that a large part of the sales in the sector is due to the different types of fermented milks, mainly yoghurts (GRANATO et al., 2010). Moreover, when developing nutritionally designed foods that promote health through gut microbial reactions, three different types of food ingredients can be used: living microorganisms (probiotics), non-digestible carbohydrates (dietary fiber and prebiotics), and bioactive plant secondary metabolites such as phenolic compounds (PUUPPONEN-PIAMIÄ *et al*., 2003). Considering that there are scarce studies that point out the effects of fruity food matrices on probiotic microbiota. The aim of this review is to provide an overview of recent studies on the interactions between the probiotic viability/activity and the food product, especially those containing a fruity base.

1.2. Gut microbiota

Components of the human microbiota and of the food entering the gut may have destructive or beneficial effects on human health.

The human gut is the natural habitat for a large and dynamic bacterial community, but a substantial part of it is still uncharacterized or unculturable (MACFARLANE & MACFARLANE, 2004; ECKBURG *et al*., 2005; FRANK & PACE, 2008; TURRONI *et al.*, 2008). Gut microbiota within a given individual are remarkably stable, although major differences may exist among different persons. The colon is certainly a host to a stable ecosystem, whereas the ecosystem of the small intestine is less stable and more susceptible to modifications (BEZKOROVAINY, 2001).

Recently the expressions 'normobiosis' and to 'dysbiosis' are well defined and allow for better understanding of microbiota behavior. Normobiosis characterizes a composition of the gut 'ecosystem' in which microorganisms with potential health benefits predominate in number over

potentially harmful ones, in contrast to dysbiosis, in which one or a few potentially harmful microorganisms are dominant, thus creating a disease-prone situation (ROBERFROID *et al.*, 2010).

The significance and effect of resident bacteria on a host's physiology and pathology have been well documented. Major functions of the gut microbiota include metabolic activities that result in save of energy and absorbable nutrients, important trophic effects on intestinal epithelia and on immune structure and function (PERDIGON *et al*., 2002; ISOLAURI, 2004; KELLY *et al.*, 2005), and protection of the colonized host against invasion by foreign microbes (GUARNER & MALAGELADA, 2003). Gut microbiota might also be an essential factor in certain pathological disorders, including multisystem organ failure, colon cancer, inflammatory bowel diseases (SALONEN *et al.*, 2010), and recently there are signs that it acts in obesity (ISOLAURI *et al.*, 2009; DIBAISE *et al.*, 2008; FURTADO *et al*., 2010; SCARPELLINI *et al.*, 2010). Probiotics have been identified as promoters of the host well-being, in spite of the inconsistency in reporting the results, which may be due to differences in the strains used, in routes of administration and doses (ADOLFSSON *et al.*, 2004).

1.3. Probiotics and prebiotics

A probiotic is a viable microbial dietary supplement that beneficially influences the host through its effects in the gut (FAO/WHO, 2002). On the other hand, prebiotics are products that strengthen normobiosis by a) selective modification in the gut microbiota's composition, b) improving feces formation by increasing the water-holding capacity and gelification of the faecal material, c) beneficial physiological effects either in the colon or the extra-intestinal compartments or d) reduction of the risk of dysbiosis and associated intestinal and systemic pathologies (ROBERFROID *et al.*, 2010).

Probiotics and prebiotics are known to have a role in prevention or treatment of some diseases (GUARNER & MALAGELADA, 2003; GAREAU *et al*., 2010). Only little changes are noticed in the microbial profile of the feces of children and elderly as the result of probiotics daily intake. However, when applied to pathologic situations, they are often sufficient to beneficially alter the course of disease. In most situations, probiotic administration results in an increase in fecal counts of bifidobacteria and lactobacilli, a decrease in fecal pH, a decline in those bacterial enzyme activities that are associated with the development of colon cancer, and then in beneficial effects in many diseases (BEZKOROVAINY, 2001; COLLADO *et al*., 2009; GAREAU *et al.*, 2010).

There are many factors that impact on the survival of ingested probiotics in the gastrointestinal tract as the level of stomach acidity, the time of exposure to acid, the concentration of bile salts and time of exposure to them, the level of bile salt hydrolase activity, and the probiotic species and strains used. Nevertheless, many probiotic strains can withstand the rigors of passage through the upper gastrointestinal tract and enter the colon in a viable state in sufficient number to affect the micro ecology and metabolism in the colon. Moreover, there are evidences *in vivo* that some bacteria strains adhere to intestinal mucosal cells, avoiding that pathogens exert their deleterious activity in a significant extent, but colonization may be unnecessary to achieve positive results in probiotic therapy. When probiotic administration stops, they are no longer recovered in a feces, which indicates that, although not colonizing, these bacteria continue to be metabolically active, thus providing health benefits to their hosts. Thus, to assure the beneficial effect, regular ingestion is recommended, even though, apparently, culturable stability data alone do not give a sufficient accurate prediction of the probiotic functionality under adverse conditions (e.g. survival under acidic and bile stress). In spite of having no agreement about the effective dose, many authors have been suggesting a minimum dose between 10^6-10^9 CFU/day to assure the therapeutic effect (VASILJEVIC & SHAH, 2008).

The enumeration of probiotic bacteria from yoghurt-like products is a challenge due to the presence also of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *Bulgaricus*. Considering the probiotic bacteria found in commercial fermented milks, the most usual selective medium for their enumeration in yoghurt-like products are: (a) deMan Rogosa Sharp (MRS) agar, at pH 6.2, with 10 μL mL^{-1} clindamycin, for *Lactobacillus acidophilus* or with 0.5 μL mL^{-1} of vancomycin for *Lactobacillus rhamnosus*, (b) LC agar for *Lactobacillus casei* which was developed by RAVULA & SHAH (1998), (c) Reinforced Clostridial Agar (RCA) with 5% of cystein, at pH 7.1, plus 100 μL mL^{-1} dicloxacillin for bifidobacteria (LANKAPUTHRA & SHAH, 1996; DAVE & SHAH, 1996; THARMARAJ & SHAH, 2003). Probiotic microorganisms have a satisfactory growth under anaerobic conditions at 37°C and can be enumerated after 48 – 72 h.

1.4. Dairy products

There are evidences that the food matrices play an important role in the beneficial health effects of probiotics on the host. Research now focuses both on characterizing specific probiotic strains and how the food matrix and the dietary content interact with the most efficient probiotic

strains (ISOLAURI, 2007). When choosing a cryoprotectant for a probiotic, also the stability in target food applications should be considered (SAARELA *et al.*, 2006).

Yoghurt-like products is considered the best-known food vehicle for probiotics because, beyond its own physico-chemical and functional characteristics, the beneficial effects of LAB present in the fermented milk are associated with health by the consumers so far (VASILJEVIC & SHAH, 2008).

Briefly, yoghurt is defined as a coagulated milk product that results from fermentation of milk by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (LAB). The conversion of lactose into lactic acid lowers the pH and, consequently, favors the precipitation of milk proteins. When the desired pH (usually around 4.5-4.7) is reached, the gel is broken by passing it through a filter system and immediately cooled to 20°C, which slows down the fermentation and facilitates the transfer of yoghurt to the package and limits changes in the structure of the gel. The product is then cooled to 4°C and maintained at this temperature during storage, transport and distribution. Though *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* cannot be considered probiotics according to FAO/WHO (2002), the influence of fruity matrix on these microorganisms will be refereed in this review, since they are often associated with probiotic strains in food products and in clinical trials (MERENSTEIN *et al*., 2010; SIMRÉN *et al*., 2010).

Several LAB species are currently used in the manufacture of yoghurt including probiotics, and supplementation of milk with functional ingredients is proposed to develop new yoghurts.

However, although the fruit-added fermented milks are responsible for more than a half of the yoghurt-like products market, only a few publications on these dairy products enriched with fruity bases are available in literature, and data regarding the possible impact of these fruits on the viability of the probiotic microorganism in the food product and in the consumer microbiota are scarce (VINDEROLA *et al.*, 2002). One of the main concerns in the probiotic fruit yoghurt production is the acidic environment that most of the fruits may confer to the product (KAILASAPATHY *et al.*, 2008).

The viability of strains of *L. acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in stirred yoghurts with fruit preparations of mango, mixed berry, passion fruit and strawberry was evaluated during shelf-life (GODWARD *et al.*, 2000; KAILASAPATHY *et al.,* 2008). The authors observed that the addition of any of the fruit preparations in different concentrations had no effect on the counts of the two probiotics tested. Similar findings were reported by Bakirci and Kavaz (2008), who observed that the counts of *S. thermophilus, L. acidophilus* and *Bifidobacterium* sp. Did not exhibit any significant difference in fermented milks supplemented with banana puree, compared to

the control. On the other hand, Donkor *et al.* (2007) observed a reduction of viable population of *Bifidobacterium animalis* Bb-12, *L. acidophilus* La-5 and *L. rhamnosus* GG during 28 days-cold storage in commercial fermented milks, as a consequence of pH decrease due to lactic and acetic acids and acids derived from fruit juice flavourings (Table 1.1).

Strains of *L. acidophilus* and *Bifidobacterium bifidum* were able to maintain their viability in yoghurt containing açai pulp at different concentrations (ALMEIDA *et al*., 2008). In a different approach, Espirito-Santo *et al*. (2010) observed that the addition of 7% of açai pulp prior to the fermentation of milk increased the monounsaturated and polyunsaturated fatty acid contents and enhanced the production of α-linolenic and conjugated linoleic acids in the probiotic yoghurt. Moreover, açai pulp favored the viability of *L. acidophilus*, *B. animalis* ssp. *lactis* and *Bifidobacterium longum* throughout 4 weeks of cold storage (ESPIRITO-SANTO *et al.*, 2010).

The fermented milks supplemented with lemon and orange fibers increased the counts of *L. acidophilus* and *L. casei* during cold storage compared to the control, but it was not so for *B. bifidum*, possibly owing to the well-known sensitivity of bifidobacteria species to the acidic environment (SENDRA *et al.*, 2008).

The association of *Saccharomyces boulardii* with yoghurt bacteria has been suggested as a factor stimulating the viability of probiotic bacteria. Lourens-Hattingh & Viljoen (2001), who investigated the ability of this probiotic yeast to survive in fermented milk with and without a "fruit mix", found higher counts in the fruit-based products likely because of the presence of sucrose and fructose in the fruit, being this yeast unable to ferment lactose.

Table 1.1 provides a literature survey on the influence of fruit addition to food products on the probiotic viability.

1.5. Nondairy products

The growing demand for new probiotic foods worldwide has stimulated the development also of nondairy products, mainly exploring fruit juice as a medium for probiotics (LUCKOW & DELAHUNTY, 2004). In this sense, VINDEROLA *et al.* (2002) observed that the natural fruit juices (green apple, kiwi, pineapple, peach and strawberry) added to growth liquid media exerted an inhibitory effect on *S. thermophilus.* Strawberry juice inhibited all probiotic strains except *L. casei,* whereas the pineapple and kiwi juices had a negative effect on the growth of *L. acidophilus* strains. Moreover, green apple juice inhibited *Lactococcus lactis* growth, whereas peach juice had no effect

on any probiotic (VINDEROLA *et al*., 2002). Sheehan *et al.* (2007), testing the viability of some lactobacilli and bifidobacteria strains in orange, pineapple and cranberry fermented juices, observed that *L. casei*, *L. rhamnosus* and *Lactobacillus paracasei* displayed the lowest sensitivity to the acidic environment of the juices, while all probiotics showed higher counts in orange and pineapple juices compared to cranberry juice.

The counts of nine lactobacilli strains added to a commercial fruit drink (made with a fruit blend) were evaluated during 80 days of cold storage, and the resistance to simulated gastro-intestinal conditions were assessed in four of them (CHAMPAGNE & GARDNER, 2008). The probiotic viability was shown to be strain-dependent and, in general, *L. rhamnosus* was more resistant than *L. acidophilus*. The data also suggested that a month of storage in the fruit blend drink had no significant effect on the sensitivity of probiotics to bile or pancreatic enzymes.

The pomegranate juice was successfully fermented by *Lactobacillus plantarum* and *L. delbrueckii* that were capable to survive well during the first two weeks, whereas *L. paracasei* and *L. acidophilus* lost their viability (MOUSAVI *et al.*, 2010). Nevertheless, as far as the influence of pomegranate tannins on the viability of probiotic bacteria present in the human intestinal microbiota is concerned, it was observed that lactobacilli and bifidobacteria were not affected by ellagitannins. Moreover, the addition of a byproduct of pomegranate juice making significantly stimulated the growth of *Bifidobacterium breve* and *Bifidobacterium infantis*, while punicalagins inhibited the one of pathogenic *Clostridium* sp. and *Staphyloccocus aureus* (BIALONSKA *et al*., 2009).

Tomato and beetroot juices proved to be potential substrates for probiotic lactic acid bacteria such as *L. acidophilus*, *L. casei*, *L. plantarum* and *L. brevis*, as all these microorganisms were capable to produce lactic acid and maintain desirable viable cell counts above 10^6 CFU/mL during shelf-life (YOON *et al*, 2004; YOON *et al*, 2005; KLEWICKA *et al*., 2009). Moreover, beetroot juice fermented by *L. casei* and *L. brevis* proved to be beneficial for the caecum microbiota activity of rats, for this probiotic food increased both the amounts of short chain fatty acids and the counts of beneficial bacteria such as *Lactobacillus* sp., *Bifidobacterium* sp., *Bacteriodes* sp., and *Enterococcus* sp., and reduced the Enterobacteriaceae population (KLEWICKA *et al.*, 2009).

The juice made of a blend of carrots, celery and apples also showed to be a good matrix for the growth of *L. acidophilus* (NICOLESCU & BURULEANU, 2010). On the other hand, the carrot juice alone demonstrated to be a suitable base for *B. lactis* and *B. bifidum* strains, but a 45% decrease in the carotenoids contents was promoted by these microorganisms (KUN *et al.*, 2008). However, *L. rhamnosus* and *L. bulgaricus* strains added to carrot juice, with or without inulin and

fructooligosaccharides, demonstrated good viability independently of the prebiotic added (NAZZARO *et al.*, 2008). In addition, the β-carotene content and antioxidant activity were preserved during one month of cold storage, thereby indicating that these nutritional components are not metabolized by *Lactobacillus* spp.. The observations of Kun *et al.* (2008) and Nazzaro *et al.* (2008) suggest the hypothesis that there is a possible relation between the probiotic strain and the content of carotenoids and antioxidant power.

Some studies pointed to the applicability of apple as an ingredient to improve probiotic viability in foods. Pieces of apple and pear were shown to offer a proper material for *L. casei* immobilization, being the probiotic bacteria nested in the cellulosic structure of these fruits resistant to the cheese making and ripening processes. As cellulose is not digested, Kourkoutas *et al.* (2006) pointed out a possible protective effect of fruit pieces during the transit through the intestinal tract, which may help *L. casei* to reach the colon. In other studies, *L. rhamnosus* also demonstrated good adherence to the surface of apple wedges (RÖßLE *et al.*, 2010; ALEGRE *et al.*, 2011). Although the probiotic activity of *L. rhamnosus* adhered to the surface of apple wedges is not guaranteed over 14 days of shelf life, it proved to be effective to reduce *Listeria monocytogenes* growth (ALEGRE *et al.*, 2011). Apple pieces demonstrated to be also able to immobilize the potentially probiotic yeast *Saccharomyces cerevisiae* for grape fermentation and to maintain its viability for more than four months under freeze storage, without any decrease of its activity (KOURKOUTAS *et al.*, 2006).

Furthermore, Osman *et al.* (2008) studied the anti-inflammatory activity of blueberry and some probiotic strains in a colitis model in rats. The authors reported that the counts of Enterobacteriaceae in the caecum decreased significantly in the group that received blueberry with and without probiotics. The concentration of short chain fatty acids in caecum was significantly higher in the *L. plantarum* group, while the *L. fermentum* one promoted the highest concentration of lactic acid compared with all other groups. Moreover, the combinations of blueberry and *L. plantarum* or *L. fermentum* were able to reduce bowel inflammation (OSMAN *et al.*, 2008).

Polyphenols such as flavonoids are ubiquitous in fruits and vegetables. Parkar *et al.* (2008) reported that the flavonoids – naringenin, rutin, phloridzin and quercetin – may have effects on gut microbiota, among which: a) an increase in the count of beneficial bacteria such as *L. rhamnosus,* b) enhanced adherence of this bacterium on the gut wall, and c) inhibition of the proliferation of enteropathogens like *S. aureus* or *Salmonella typhimurium*. Besides, flavonoids can suffer some structural modifications when submitted to fermentation. For instance, Bisakowski *et al.* (2007) evaluated the flavonoidic profile of red onions fermented by *L. plantarum* and found alterations on the quercetin glucosides profile. After fermentation, the amounts of quercetin diglucoside and

monoglucoside were significantly increased, which was pointed out by the authors as an advantage, having these glicosides well-recognized antioxidant activity.

The ingestion of DF can modulate the intestinal microbiota, which is able to ferment the indigestible fibers mainly into short-chain fatty acids that are absorbed by the colonocytes stimulating water and Na absorption (SEMBRIES *et al.*, 2003). These effects were demonstrated in the study of Wang *et al.* (2007) who observed that a diet based on the Taiwanese yam (a fiber-rich tubercle) increased the faecal mass and short-chain fatty acids output, increased bifidobacteria counts and decreased *Clostridium perfringens* counts in BALB/c mice faeces. Another example of the gut microecology modulation by DF intake is provided in the work of Foo *et al.* (2008), who observed in the faeces of rats fed with a fiber-rich compost made of fermented fruits (lime, sugar cane and rice bran) reduction counts of Enterobacteriaceae and increased counts of beneficial LAB, whereas the decrease in plasma cholesterol concentration can be considered an important extra benefit. Costabile *et al.* (2008) observed that the counts of bifidobacteria and lactobacilli in human faeces were significantly higher in the group that received dietary intake of plain-grain compared to the one that received wheat bran, which demonstrates the importance of the type of dietary fiber for the gut microbiota formation.

From the technological point of view, dietary fibers have been employing as functional ingredient thanks to their prebiotic activity. Among four different dehydrated prebiotic fibers - inulin, oat bran, unripe banana flour and apple fiber, used to immobilize *L. casei*, the oat bran was the one that promoted the greatest viability of this probiotic during cold storage (GUERGOLETTO *et al.*, 2010). In another study with soluble fibers, the pitaya (dragon fruit) oligosaccharides were shown to be resistant to hydrolysis by gastric juice and α-amylase and demonstrated to have a prebiotic effect on *B. bifidum* and *L. delbrueckii* strains (WICHIENCHOT *et al.*, 2010).

1.6. Other products

Besides fermented milks, fruits and fruit juices, a wide variety of foods have been tested as a vehicle for the intake of different probiotic strains. For instance, fermented acerola (*Malpighia emarginata*) ice cream demonstrated to support probiotic bacteria viability, since the counts of *B. longum* and *B. lactis* kept above 10^6 CFU/g during 15 weeks storage even in acidic products (pH 4.5) (FAVARO-TRINDADE *et al.*, 2006). The counts of *B. lactis* and *L. paracasei* remained above 10^7 CFU/g in the coconut flan during shelf-life (CORREA *et al.*, 2008).

In a recent study, pear puree fermented by *Leuconostoc mesenteroides* was able to maintain constant probiotic counts around 10^9 CFU/g for 14 days (KIM *et al.*, 2010). Banana puree was fermented by microencapsulated *L. acidophilus* resulting in a symbiotic product with good consumer acceptance and desirable probiotic cell counts (TSEN *et al.*, 2004). *L. plantarum* showed viability in barley, wheat and malt extracts, showing the highest counts in the last medium (CHARALAMPOPOULOS & PANDIELLA, 2010). The daily intake of a coffee mix drink containing mannooligosaccharides significantly increased the counts of *Bifidobacterium* sp. in human fecal microbiota (UMEMURAS *et al.*, 2004).

Cheese offers an attractive food-based delivery vehicle for probiotic cultures and biogenic substances such as conjugated linoleic acid (CLA) and bioactive peptides. Compared with many other fermented foods, it has relatively high pH and fat content, a solid consistency and a higher buffering capacity (HAYES *et al.*, 2006). Saxelin *et al.* (2010), comparing the recovery of a combination of *L. rhamnosus* GG and LC705, *Propionibacterium freudenreichii* subsp. *shermanii* JS, and *B. animalis* subsp. *lactis* Bb12 from feces of volunteers who had received those probiotics in capsules, fermented milk or cheese, found that the quantity of *L. rhamnosus* strains were not affected by the administration matrix. On the other hand, when consumed in cheese, the faecal counts of bifidobacteria and surprisingly of propionibacteria were lower.

Finally, Possemiers *et al.* (2010) evaluated chocolate as a potential protective carrier for oral delivery of a microencapsulated mixture of *Lactobacillus helveticus* CNCM I-1722 and *B. longum* CNCM I-3470. Data indicate that the coating of the probiotics in chocolate is an excellent solution to protect them against environmental stress conditions and for optimal delivery.

Table 1.1. Literature survey on the influence of the fruity food matrix on the viability of probiotics and others microorganisms commonly associated to them.

Microorganism[*, **]	**Food product**	**Fruit** [**]	**General effect of the fruit food base on microorganism viability regarding the other treatments**	**Reference**
Lactobacillus acidophilus LAFTI L10 *Bifidobacterium animalis ssp. lactis* LAFTI B94	Fermented milk	I) Passion fruit II) Mango III) Mixed berries IV) Strawberry	I) + (except for *L. acidophilus*) II) + III) + (except for *L. acidophilus*) IV) +	(KAILASAPATHY *et al.*, 2008)
I) *Streptococcus thermophilus* II) *L. acidophilus* III) *Bifidobacterium* sp.	Fermented milk	Banana	I) none II) none III) none	(BAKIRCI & KAVAZ, 2008)
I) *L. acidophilus* II) *Bifidobacterium bifidum*	Fermented milk	Açai	I) + II) +	(ALMEIDA *et al.*, 2008)
I) *L. acidophilus* L10 II) *B. animalis subsp. lactis* Bl04 III) *B. animalis subsp. lactis* B94 IV) *Bifidobacterium longum* Bl05	Fermented milk	Açai	I) + II) + III) none IV) +	(ESPIRITO-SANTO *et al.*, 2010)
Bifidobacterium animalis Bb-12 *Lactobacillus acidophilus* La-5 *Lactobacillus rhamnosus* GG *Streptococcus thermophilus* *Lactobacillus delbrueckii* ssp. *bulgaricus*	Fermented milk	I) Passion fruit II) Mango III) Apricot/mango/peach blend	I) + II) + III) +	(DONKOR *et al,*. 2007)
I) *L. acidophilus* CECT 903 II) *Lactobacillus casei* CECT 475 III) *Bifidobacterium bifidum* CECT 870	Fermented milk	Fibers of orange and lemon	I) + II) + III) -	(SENDRA *et al.*, 2008)
I) *Saccharomyces boulardii* ATCC 74012	Fermented milk	Fruit blend	I) +	(LOURENS-HATTINGH & VILJOEN, 2001)
L. acidophilus *L. casei group* *Lactobacillus rhamnosus* *Lactococcus lactis* *B. longum*	Juice	I) green apple II) kiwi III) pineapple IV) peach V) strawberry	I) + (except for *L. lactis*) II) + (except for *L. acidophilus*) III) + (except for *L. acidophilus*) IV) + V) - (except for *L. casei*)	(VINDEROLA *et al.*, 2002)

Table 1.1 (*cont.*)

Microorganism[*,**]	**Food product**	**Fruit** [**]	**General effect of the fruit food base on microorganism viability regarding the other treatments**	**Reference**
Lactobacillus salivarius ssp. salivarius UCC118 *L. salivarius ssp. salivarius* UCC500 *L. paracasei ssp. paracasei* NFBC43338 *L. rhamnosus* GG *L. casei* DN- 114 001 *B. animalis ssp. lactis* Bb-12	Juice	I) orange, II) pineapple III) cranberry	I) + II) + III) -	(SHEEHAN *et al.*, 2007)
I) *L. acidophilus* II) *Lactobacillus brevis* III) *L. rhamnosus* IV) *Lactobacillus fermentum* V) *Lactobacillus plantarum* VI) *Lactobacillus reuteri*	Juice	Blend of pineapple, apple, orange, pear and/or grape, passion fruit, lemon and purees (peach, strawberry, mango and kiwi)	I) - II) + III) + IV) + V) + VI) +	(CHAMPAGNE & GARDNER, 2008)
I) *L. rhamnosus* R0011	Juice	Blend of apple, pear and raspberry	I) +	(CHAMPAGNE & GARDNER, 2008)
I) *L. acidophilus* DSMZ 20079 II) *L. plantarum* DSMZ 20174 III) *L. delbrueckii* DSMZ 20006 IV) *L. paracasei* DSMZ 15996	Juice	Pomegranate	I) – II) + III) + IV) –	(MOUSAVI *et al.*, 2010)
I) *L. acidophilus* LA39 II) *L. casei* A4 III) *L. delbrueckii* D7 IV) *L. plantarum* C*3*	Juice	Tomato Beetroot	I) + II) + III) + IV) +	(YOON *et al.*, 2004; YOON *et al*, 2005)
L. acidophyllus LA-5	Juice	Carrot and apple Carrot, apple and celery	I) + II) +	(NICOLESCU & BURULEANU, 2010)

Table 1.1 (*cont.*)

Microorganism[*,**]	**Food product**	**Fruit** [**]	**General effect of the fruit food base on microorganism viability regarding the other treatments**	**Reference**
I) *B. animalis ssp. lactis* Bb-12 II) *B. bifidum* (isolated from human faeces)	Juice	Carrot	I) + II) +	(KUN *et al.*, 2008)
I) *Lactobacillus delbrueckii subsp. bulgaricus* DSM 20 081 II) *L. rhamnosus* DSM 20 711	Juice	Carrot	I) + II) +	(NAZZARO *et al.*, 2008)
I) *Saccharomyces cerevisiae* II) *Kluyveromyces marxianus* III) *Lactobacillus casei*	Pieces of fruit for microorganism immobilization	Apple	I) + II) + III) +	(KOURKOUTAS *et al.*, 2006)
I) *L. rhamnosus* GG LGG	Pieces of fruit for probiotic immobilization	Apple	I) +	(RÖßLE *et al.*, 2010) (ALEGRE *et al.*, 2011)
I) *Lactobacillus plantarum* S1	Fermented vegetable	Onions	I) +	(BISAKOWSKI *et al.*, 2007)
I) *Lactobacillus casei* LC-1	Cereal / fiber for probiotic immobilization	I) oat bran II) banana flour III) apple fiber	I) ++ II) + III) +	(GUERGOLETTO *et al.*, 2010)
I) *Bifidobacterium animalis ssp. lactis* II) *Bifidobacterium longum*	Ice cream	Acerola	I) + II) +	(FAVARO-TRINDADE *et al.*, 2006)
I) *Bifidobacterium animalis ssp. lactis* BL04 II) *Lactobacillus paracasei ssp. paracasei* LBC 82	Flan	Coconut	I) + II) +	(CORREA *et al.*, 2008)
I) *Leuconostoc mesenteroides*	Puree	Pear	I) +	(KIM *et al.*, 2010)

Table 1.1 (*cont.*)

Microorganism[*,**]	**Food product**	**Fruit** [**]	**General effect of the fruit food base on microorganism viability regarding the other treatments**	**Reference**
I) *L. acidophilus* CCRC 10695 II) *L. acidophilus* CCRC 10695 immobilized by κ-carrageenan	Puree	Banana	I) None II) +	(TSEN *et al.*, 2004)
I) *Lactobacillus helveticus* CNCM I-1722 II) *Bifidobacterium longum* CNCM I-3470	Chocolate	Cacao	I) + II) +	(POSSEMIERS *et al.*, 2010)

* Some microorganisms' species were also reported with the strain identification in the study.

** The Roman numerals indicate correspondence between the variable and the effect on microorganism viability on the 4th column.

Abbreviations: (+) a significant positive effect regarding the others treatments and/or the control; (++) an even more significant positive effect regarding the others treatments; (-) a significant negative effect regarding the others treatments and/or the control.

CHAPTER II

AÇAI PULP ADDITION IMPROVES FATTY ACID PROFILE AND PROBIOTIC VIABILITY IN YOGURT

2.1. Introduction

The consumption of regional foods is recommended by the Food and Agriculture Organization of the United Nations (FAO, 2008) for the sustainable development of indigenous and non-indigenous farming communities in areas of high biological diversity. A good incentive for the necessary integration between socio-economic and environmental interests involving regional food is the development of new food products with native fruits. The açai fruit (*Euterpe oleracea*, Mar., Arecaceae) is important for sustainable agribusiness in the Amazon region, where it is native (MUÑIZ-MIRET *et al.,* 1996; SILVA *et al.,* 2006).

Reports on biological activities of açai fruit include antioxidant activity and reduction in blood pressure in hypertensive rats (LICHTENTHÄLER *et al.,* 2005; MERTENS-TALCOTT *et al*., 2008), especially due to its high anthocyanins content. Moreover, the açai pulp oil could be compared to hazelnut, olive (BENITEZ-SÁNCHEZ *et al.,* 2003) and avocado (NASCIMENTO *et al*., 2008) oils, which are rich in monounsaturated 60% and polyunsaturated 14% fatty acids (MENEZES *et al.,* 2008). This fact encourages the use of açai as an essential fatty acid source, according to the most recent US food pyramid (EKINCI *et al.,* 2008). However the açai fatty acid profile has been under-exploited, particularly in the development of new food products.

Due to the expanding market of dairy companies, there has been a merging of dairy product and fruit beverage markets, with the introduction of hybrid dairy products, such as 'juiceceuticals', offering health, flavour and convenience (KHURANA & KANAWJIA, 2007). The addition of açai pulp can potentially enhance the functional properties of probiotic yogurt (ALMEIDA *et al*., 2008). Probiotics are defined as live microorganisms that are able to colonize the gastrointestinal tract, and when administered in adequate amounts (FAO/WHO, 2001), confers a health benefit on the host beyond inherent general nutrition (such as traveler's diarrhea prevention, lowering serum cholesterol, duration of rotavirus diarrhea reduction, immune system stimulation and colon cancer prevention (FARNWORTH, 2008).

During the fermentation of milk, fatty acid profiles progressively change as a result of microbial growth (EKINCI *et al.,* 2008). The production of free fatty acids by lactic acid bacteria (LAB) through lipolysis of milk fat has been reported by Coskun and Ondul (2004) and Yadav *et al.* (2007). Kankaanpäa et al. (2004) showed that free polyunsaturated fatty acids (PUFA) in the growth medium of lactobacilli may induce changes in fatty acids, such as the degree of fatty acid unsaturation, cyclization, and proportions of PUFA containing 18 carbons with conjugated double bonds, such as conjugated linoleic acid (CLA).

Studies show that CLA can be produced by some strains of lactobacilli, bifidobacteria and propionibacteria from linoleic acid (OGAWA *et al.,* 2005; YADAV *et al*., 2007; EKINCI *et al.,* 2008). Alonso *et al*. (2003), Prandini *et al.* (2007) and Ekinci *et al*. (2008) reported health benefits attributed to CLA, such as protection against arteriosclerosis, modulation of immune system, and body fat reduction.

Moreover, Das and Fams (2002) observed that long chain polyunsaturated fatty acids (LCPUFAs) and have similar beneficial actions, such as the ability to restore normal and healthy gut microecology. Furthermore, both probiotics and LCPUFAs have anti-inflammatory actions alleviating changes related to allergic inflammation (DAS & FAMS, 2002). In addition, as observed by Kankaanpäa *et al.* (2001), LCPUFAs, especially α-linolenic acid, promote adhesion of *Lactobacillus casei* to mucosal surfaces, potentiating the beneficial actions of *Lactobacilli*.

Kim and Liu (2002) reported that the addition of sunflower oil increased CLA content by LAB in dairy products. Therefore, the addition of açai pulp to skim milk may increase the functional aspects of the probiotic yogurt, since it increases the content of long chain mono-and polyunsaturated fatty acids.

The objective of the present study was to evaluate the effect of açai pulp addition on fatty acid profile and probiotic viability in stirred yogurts. Moreover, the effect of açai pulp on parameters such fermentation time, pH, titratable acidity and lactose content were also examined.

2.2. Materials and methods

2.2.1. Fruit pulp

Açai pulp (pH measured to be 4.90) was obtained from a local market at Jundiaí, Brazil. The fruit pulp was homogenized and then lyophilized in a freeze dryer (Lyolab-G, LSL Secfroid, Lausanne, Switzerland) and stored at -20ºC until use. In order to facilitate the mixture of açai pulp into the reconstituted milk, the particle size of freeze dried pulp was reduced to less than 42 μm,

measured through sieves (Granutest, Sao Paulo, Brazil) with mesh diameters of 200 μm, 119 μm, 59 μm and 42 μm.

2.2.2. Milk preparation

Skim milk powder (2 g fat L^{-1}; Molico, Nestlé, Araçatuba, Brazil) was reconstituted to 12% (w/w) in distilled water and divided into two milk samples. Freeze dried açai pulp was added to one sample at 7 g 100 g^{-1} w/w (YA). This amount of fruit pulp was chosen because it was the highest level of fruit addition that still resulted in texture expected by consumers for a spoonable yogurt (data not shown). The other milk sample was used as control, i.e., without the addition of açai pulp (Y). Both milk bases were thermally treated at 85ºC for 15 min under agitation in a water bath and then portioned into sterile Schott flasks (500 mL), cooled in an ice bath, and stored at 4ºC for 24 h.

2.2.3. Microbial cultures

The freeze-dried starter yogurt culture (CY340, DSM, Moorebank, NSW, Australia) - composed of *Streptococcus thermophilus* (St) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb) - and four probiotic cultures: *Lactobacillus acidophilus* (L10, DSM), *Bifidobacterium animalis* subsp. *lactis* (BL04, Danisco, Madison, WI, USA, and B94, DSM), and *B. longum* (BL05, Danisco) were used in this study. The lyophilized culture was diluted in milk (sterilized at 121ºC for 15 min) and divided into aliquots into Eppendorf® flasks and frozen at -20 °C. Before fermentation, the cultures in the Eppendorf® flasks were thawed and diluted with 50 mL sterilized milk (inoculum). Each Schott® flask containing 500 mL of reconstituted milk was inoculated with 1 mL of yogurt starter culture (St-Lb, CY340) and 1 mL of probiotic culture.

2.2.4. Experimental procedure

Ten different yogurts were prepared with açai pulp and different probiotic strains added. The experimental design is presented in Table 2.1.

Table 2.1. Experimental design to evaluate the effect of addition of açai pulp on fatty acid profile and probiotic viability in yogurt.

Micro-organisms (strain)	Açai pulp	Sample coding	[1]Counts (Log CFU mL^{-1})
			[2]Probiotic
[3]*Streptococcus thermophilus* + *Lactobacillus delbrueckii* subsp. *bulgaricus* (CY340)	-	**Y**	-
Y + *Lactobacillus acidophilus* (L10)	-	**YLa**	6.38±0.16
Y + *Bifidobacterium animalis* subsp. *lactis* (Bl04)	-	**YBl04**	6.46±0.22
Y + *Bifidobacterium longum* (Bl05)	-	**YBl05**	6.61±0.15
Y + *Bifidobacterium animalis* subsp. *lactis* (B94)	-	**YB94**	6.59±0.10
Y	+	**YA**	-
Y + *Lactobacillus acidophilus* (L10)	+	**YALa**	6.38±0.16
Y + *Bifidobacterium animalis* subsp. *lactis* (Bl04)	+	**YABl04**	6.46±0.22
Y + *Bifidobacterium longum* (Bl05)	+	**YABl05**	6.61±0.15
Y + *Bifidobacterium animalis* subsp. *lactis* (B94)	+	**YAB94**	6.59±0.10

[1]Means ± standard deviation measured in the inocula.

[2]N = 8.

[3]*Streptococcus thermophilus* (St) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb) inocula counts (N = 40) were 8.15 ± 0.21 and 4.31 ± 0.16 Log CFU mL^{-1}, respectively.

Abbreviations: without açai pulp (-); with açai pulp (+).

Immediately after inoculation, Schott® flask samples were transferred to water bath equipment assembled to a CINAC (*Cynetique d´acidification*, Ysebaert, Frépillon, France) system (SPINNLER & CORRIEU, 1989). This system allowed continuous measurement and recording of pH through a program connected to a computer. Two independent batch fermentations were carried out on different days at 42ºC up to pH 4.5 and replicated twice. After pH 4.5 was reached, the fermentation was interrupted by cooling the flasks to 20°C in an ice bath, and the fermentation time ($T_{pH4.5}$) was recorded. The coagulum was then broken by means of a perforated disk on a stainless steel rod that was moved upwards and downwards for 2 min. The stirred yoghurt was put into 50 mL polypropylene cups, thermally sealed and stored at 4°C.

2.2.5. pH, titratable acidity and lactose

The pH of fermented milk post-acidification was determined at days 1, 14 and 28 using a pH meter model Q-400M1 (Quimis, São Paulo, Brazil). Titratable acidity was analysed as recommended by AOAC (1995) and lactose content was determined by the Lane-Eynon method based on the reduction of copper (AOAC, 1995). The results were expressed as the means of four replications.

2.2.6. Microbiological analyses

Bacterial enumerations were carried out at days 1, 14 and 28 in four replicates of each batch. Samples (1 mL) were diluted with 0.1% sterile peptonated water (9 mL). Afterwards, serial dilutions were carried out, and bacteria were counted, applying the pour plate technique (Kodaka et al., 2005). All media were obtained from Oxoid (Basingstoke, UK). In co-cultures, *S. thermophilus* colonies were enumerated in M17 agar whereas Lb were counted in MRS (pH 5.4), both under aerobic incubation at 37°C for 48 h. All probiotic microorganisms were incubated under anaerobic conditions, provided by AnaeroGen (Oxoid), at 37°C for 72 h. Enumerations of *L. acidophilus* were carried out in MRS (pH 6.2) plus 10 μL mL^{-1} clindamycin, and *B. lactis* B04, B94 and *B. longum* Bl05 in Reinforced Clostridial Agar plus 100 μL mL^{-1} dicloxacillin. Antibiotics were employed to allow selective growth of bifidobacteria. M17 and MRS media (pH 5.4) were prepared according to Jordano *et al.* (1992) and Dave and Shah (1996), and MRS plus clindamycin according to Lankaputhra and Shah (1996). Cell concentration was expressed as Log CFU mL^{-1} of fermented milk.

2.2.7. Fatty acid profiles

Lipids were extracted in triplicate from yogurts with or without açai pulp according to ISO method 14156 (2001). Fatty acid methyl esters (FAMEs) were prepared by esterification according to ISO method 15884 (2002).

Analyses of FAMEs were carried out in a gas chromatograph, model 3400CX (Varian, São Paulo, Brazil) equipped with a split-injection port, a flame-ionization detector and a software package (Varian Star Chromatography Workstation version 5.5, Varian Inc., Palo Alto, CA,USA) for system control and data acquisition. Injections were made into a 30 m long fused silica capillary column with 0.25 mm internal diameter, coated with 0.25 μm of Chrompack CP-Wax 52CB (ChromTech Apple Valley, MN, USA), using helium as carrier gas at a flow rate of 1.5 mL min^{-1},

and a split ratio of 1:50. The injector temperature was set at 250ºC and the detector at 280ºC. The oven temperature was initially set at 75ºC for 3 min, then programmed to increase to 150ºC at a rate of 37.5ºC min^{-1}, and then to 215°C at a rate of 3°C min^{-1}. Samples (1 μL) were injected manually after a dwell-time of *ca* 2 s. Injections of each FAME were carried out in triplicate.

Qualitative fatty acid (FA) composition of the samples was determined by comparing the retention times of the peaks produced after injecting the methylated samples with those of the respective standards of FA (catalog# 05632, 189-19; Sigma, Bellefonte, PA, USA). The quantitative composition of each FA was calculated from the area of each peak, and expressed as a percentage according to the Official Method Ce 1-62 (AOCS, 1997). The results of all samples were reported as mean values of twelve runs. Classification of fatty acids, with respect to the chain number of carbon atoms, was according to Ackman (2007). The fatty acids were expressed as g 100 g^{-1} of total fatty acid.

2.2.8. Statistical analyses

Results were analyzed by two-way ANOVA, and the effect of time and different interactions (probiotic culture, treatment – with or without açai) were assessed using a General Linear Model with Statistica Software 8.0 (Statsoft, Tulsa, OK, USA). 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.

2.3. Results and Discussion

2.3.1. Fermentation time

Addition of açai pulp to the milk before fermentation had a significant effect on pH, reaching 6.52 (± 0.02) in skimmed milk, and 6.42 (± 0.01) in the sample with açai pulp ($P < 0.05$). Açai pulp caused a significant reduction ($P < 0.05$) of $T_{pH4.5}$ in yogurt without probiotics and in yogurt with *L. acidophilus* L10, *B. lactis* B94 and with *B. longum* Bl05 strains compared to their control without fruit, pointing to a synergistic effect of açai pulp and lactic bacteria on total fermentation time in these cases (Figure 2.1). A shorter fermentation time (~5h, $P < 0.05$), was observed in yogurts containing *Bifidobacterium animalis* subsp. *lactis* Bl04 or B94 (Figure 2.1) but in these cases, açai pulp showed no effect ($P > 0.05$) on $T_{pH4.5}$.

According to Schauss *et al* (2006), açai pulp contains 8.1 g of protein, 32.5 g of lipids, 52.2 g of carbohydrates, 44.2 g of dietary fiber and 3.8 g of ash per 100 g of freeze-dried pulp. Donkor *et al* (2007) and Oliveira *et al* (2009) reported a lower fermentation time for probiotic yogurts supplemented with prebiotics. Therefore, the presence of açai pulp, as a prebiotic, may have positively influenced the fermentation time of *L. acidophilus* L10 and *B. longum* Bl05 yogurts. The mineral content of açai pulp shows high amounts of magnesium, 124.4 mg (MENEZES *et al,* 2008), calcium, 260.0 mg (SCHAUSS *et al,* 2006) and potassium, 900 mg (Menezes et al, 2008), in addition to minor quantity of zinc, 6.0 mg and iron, 15.0 mg (SANABRIA & SANGRONIS, 2007), manganese, 17.1 mg and selenium, 0.02 mg (MENEZES *et al,* 2008) per 100 g of freeze-dried pulp. Bomba *et al* (2002) reported that some micronutrients, such Mn, are essential for bifidobacteria and lactobacilli growth. Moreover, the incorporation of micronutrients into the milk, such as peptides and amino acids, can lead to a reduction in fermentation time (OLIVEIRA *et al,* 2001).

2.3.2. pH, titratable acidity and lactose content reduction

At day 1, pH varied from 4.49 to 4.59 amongst the treatments. The açai yogurts with *L. acidophilus* L10 and *B. animalis* ssp. *lactis* Bl04 strains and without probiotic had lower pH ($P < 0.05$) in relation to the corresponding controls without pulp (Table 2.2). However, açai yogurt with B94 strain, YAB94, showed a significant ($P < 0.05$) increase in pH when compared to its control, YB94 (Table 2.2). The titratable acidity data ranged from 0.81 to 0.97 mg lactic acid g^{-1}. Higher levels ($P < 0.05$) of titratable acidity were observed at day 1 in açai yogurts with *L. acidophilus* L10, *B. animalis* ssp. *lactis* Bl04 and B94 strains compared to their respective control yogurts (Table 2.2).

At day 14, açai yogurt without probiotic (YA) showed higher ($P < 0.05$) pH compared to its control (Y) without pulp (Table 2.2). Titratable acidity was significantly higher ($P < 0.05$) in the yogurt containing açai for the YALa yogurts when compared to the controls without fruit (YLa), (Table 2.2). On the other hand, titratable acidity was lower ($P < 0.05$) in açai yogurt without probiotic (YA) compared to its control (Y), (Table 2.2). After two weeks of cold storage, yogurts with or without açai with *B. longum* Bl05, *B. animalis* ssp. *lactis* Bl04 and B94 strains showed no difference ($P > 0.05$) on titratable acidity (Table 2.2).

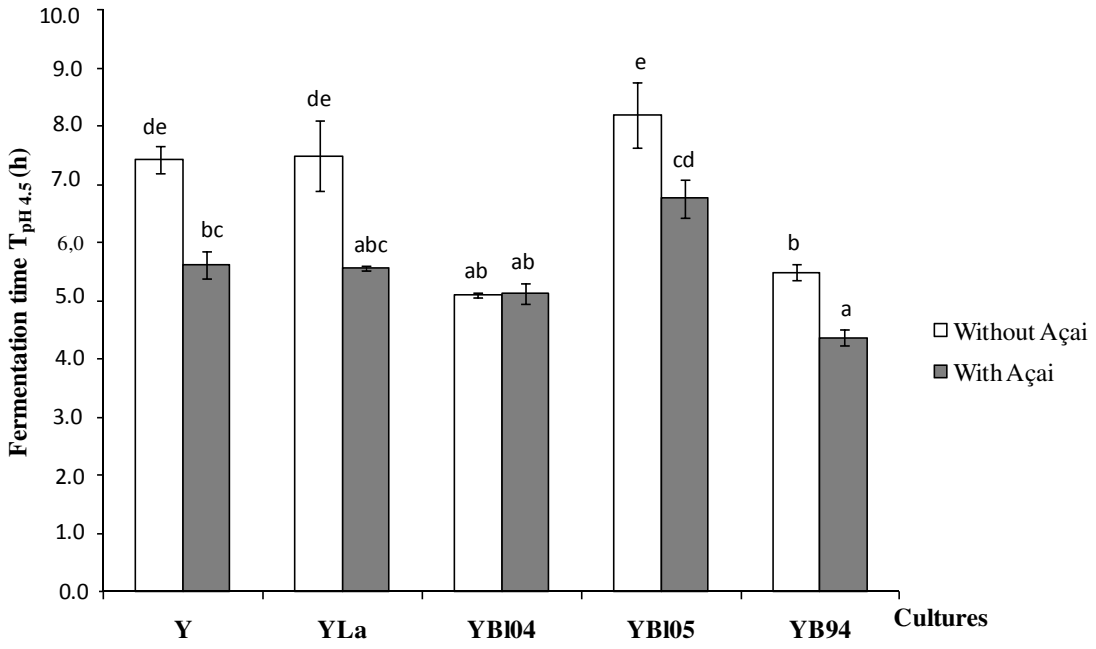

Figure 2.1. Fermentation time ($T_{pH\ 4.5}$) of yogurt with and without açai pulp by starter co-cultures associated with different probiotic strains.

Abbreviations: Yogurts associated with *L. acidophilus* L10 (YLa), *B. lactis* Bl04 (YBl04), *B. longum* Bl05 (YBl05) and *B. lactis* B94 (YB94). Means with different letters are significantly different (P<0.05). N = 4.

After 28 days of cold storage, the pH varied from 4.20 to 4.33 amongst the different treatments (Table 2.2). Açai yogurts containing Bl04 or Bl05 strains showed a significantly higher pH (P < 0.05) in relation to their respective controls without fruit (Table 2.2). Nevertheless, titratable acidity varied from 0.94 to 1.08 mg lactic acid g^{-1} amongst treatments and showed no difference (P > 0.05) due to açai presence in relation to the respective controls (Table 2.2).

Lactose content decreased after fermentation for both the original skim milk and the skim milk with açai pulp (data not shown). Lactose content of reconstituted skim milk was reduced by 18.9% by starter co-culture St-Lb CY340 and the açai yogurt showed a 16.3% decrease in lactose content by the same starter co-culture, showing a significant (P < 0.05) effect of fruit pulp addition. When the effects of açai and probiotic culture are considered together, açai yogurts containing *L. acidophilus* L10 or *B. animalis* ssp. *lactis* Bl04 showed the lowest lactose content reduction of 4.7% and 3.5% respectively (data not shown).

Table 2.2. Post-acidification (pH) and titratable acidity during shelf-life of yogurts with (YA) and without (Y) açai pulp.

Yogurts	pH *			Titratable acidity (% lactic acid) *		
	D 1	D 14	D 28	D 1	D 14	D 28
Y	4.56 ^{ef}	4.24 ^{ac}	4.25 ^{abcd}	0.85 ^{abcd}	0.99 ^{cd}	1.03 ^{bc}
YLa	4.58 ^{fg}	4.24 ^{abc}	4.23 ^{acd}	0.82 ^{ab}	0.94 ^{abc}	0.97 ^{ab}
YBl04	4.59 ^{g}	4.22 ^{c}	4.20 ^{cd}	0.84 ^{abc}	0.96 ^{bcd}	1.00 ^{abc}
YBl05	4.51 ^{abc}	4.31 ^{ab}	4.20 ^{c}	0.85 ^{abcd}	0.85 ^{e}	0.97 ^{ab}
YB94	4.49 ^{ab}	4.27 ^{abc}	4.28 ^{abe}	0.81 ^{a}	0.81 ^{ab}	0.97 ^{ab}
YA	4.50 ^{a}	4.31 ^{b}	4.31 ^{be}	0.92 ^{cde}	0.92 ^{f}	1.08 ^{c}
YALa	4.49 ^{a}	4.30 ^{ab}	4.27 ^{abde}	0.97 ^{e}	0.97 ^{d}	1.01 ^{abc}
YABl04	4.53 ^{cde}	4.27 ^{abc}	4.33 ^{e}	0.96 ^{e}	0.96 ^{d}	0.94 ^{a}
YABl05	4.53 ^{bcd}	4.29 ^{abc}	4.28 ^{abe}	0.93 ^{de}	0.93 ^{ae}	0.94 ^{a}
YAB94	4.55 ^{de}	4.31 ^{ab}	4.25 ^{abcd}	0.90 ^{bcde}	0.90 ^{abc}	0.96 ^{ab}

Means (N = 4) with different letters in the same column are significantly different (P<0.05). * Standard deviations were under 0.05.

Abbreviations: YLa and YALa yogurts associated with *L. acidophilus* L10; YBl04 and YABl04 yogurts associated with *B. lactis* Bl04; YBl05 and YABl05 yogurts associated with *B. longum* Bl05; YB94 and YAB94 yogurts associated with *B. lactis* B94.

2.3.3. Counts of viable microorganisms

Inoculation rate of starter co-culture of *S. thermophilus* - *L. delbrueckii* ssp. *bulgaricus* CY340 and probiotic strains showed no significant difference ($P > 0.05$) among treatments (Table 2.1). *S. thermophilus* counts varied from 9.6 to 10.6 Log CFU mL^{-1} after 1 day of cold storage, and showed the highest counts ($P < 0.05$) in the yogurt with *B. lactis* Bl04 strain and açai pulp (Figure 2.2). However, on day 14 all açai yogurts showed higher ($P < 0.05$) counts of *S. thermophilus* compared to yogurt without fruit. After 4 weeks of cold storage, *S. thermophilus* counts were significantly ($P < 0.05$) higher in açai yogurts with *L. acidophilus* L10, *B. lactis* Bl04 and B94 strains (Figure 2.2).

After 1 day of cold storage, *L. delbrueckii* ssp. *bulgaricus* exhibited poor growth and the counts varied from 3.0 to 5.9 Log CFU mL^{-1} (Figure 2.3). On day 14, açai yogurts without probiotic (YA) and açai yogurts with *B. lactis* Bl05 strain presented higher counts ($P < 0.05$) compared to their control without pulp. *L. acidophilus* L10 had the best positive effect ($P < 0.05$) on *L. delbrueckii ssp. bulgaricus* counts, which were ~5.9 Log CFU mL^{-1} in both yogurts with or without açai on days 1 and 14 (Figure 2.3). The counts of *L. delbrueckii* ssp. *bulgaricus* on day 28 varied from 1.0 to 1.7 Log CFU mL^{-1}. The presence of açai pulp had a positive effect ($P < 0.05$) upon the counts of this microorganism in yogurts without probiotic and in yogurts with *B. lactis* Bl05 strain; likewise on day 14.

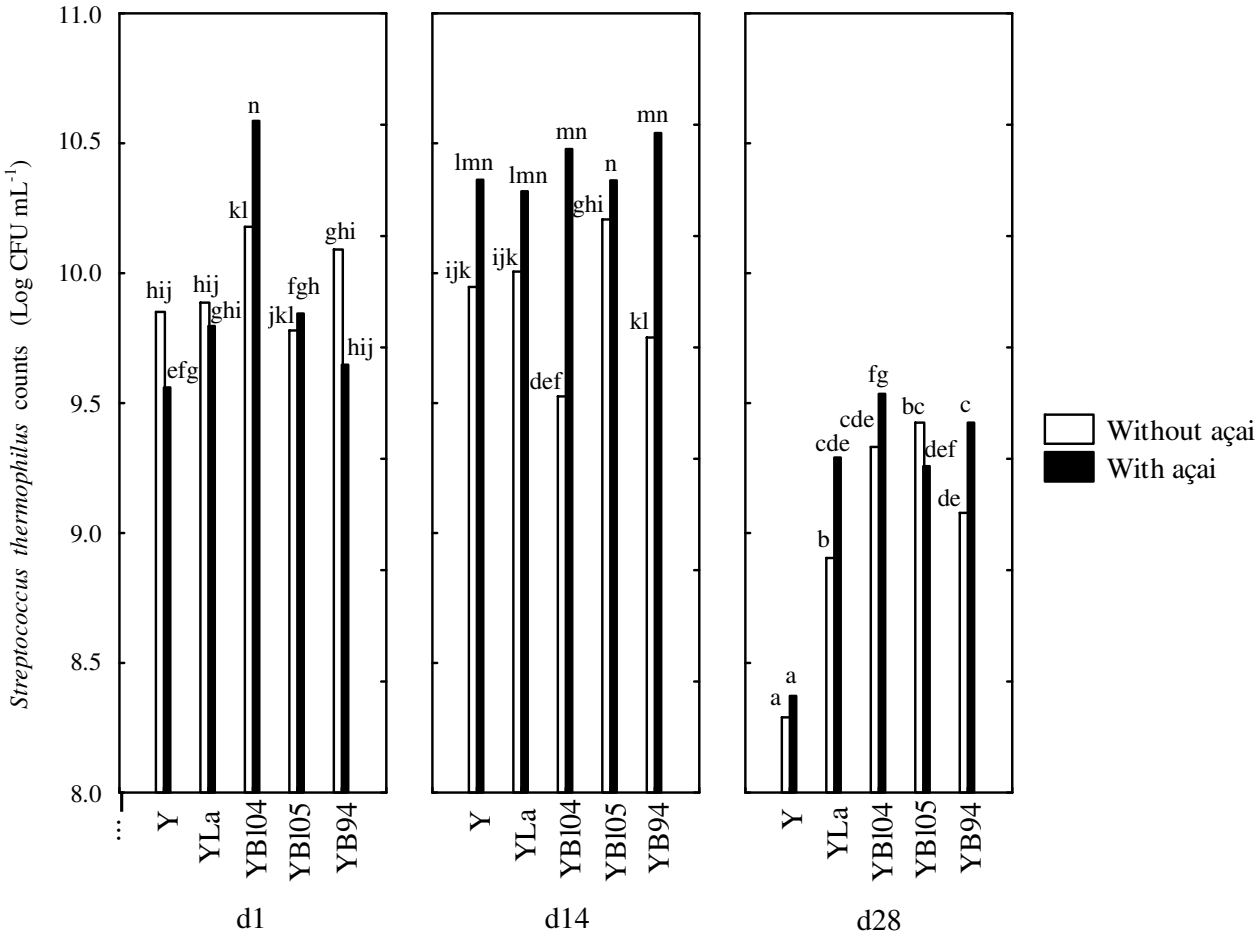

Figure 2.2. *Streptococcus thermophilus* counts in yogurt with and without açai pulp during shelf-life. Abbreviations: Yogurts associated with *L. acidophilus* L10 (YLa), *B. lactis* Bl04 (YBl04), *B. longum* Bl05 (YBl05) and *B. lactis* B94 (YB94). Means with different letters are significantly ($P < 0.05$) different. N = 40.

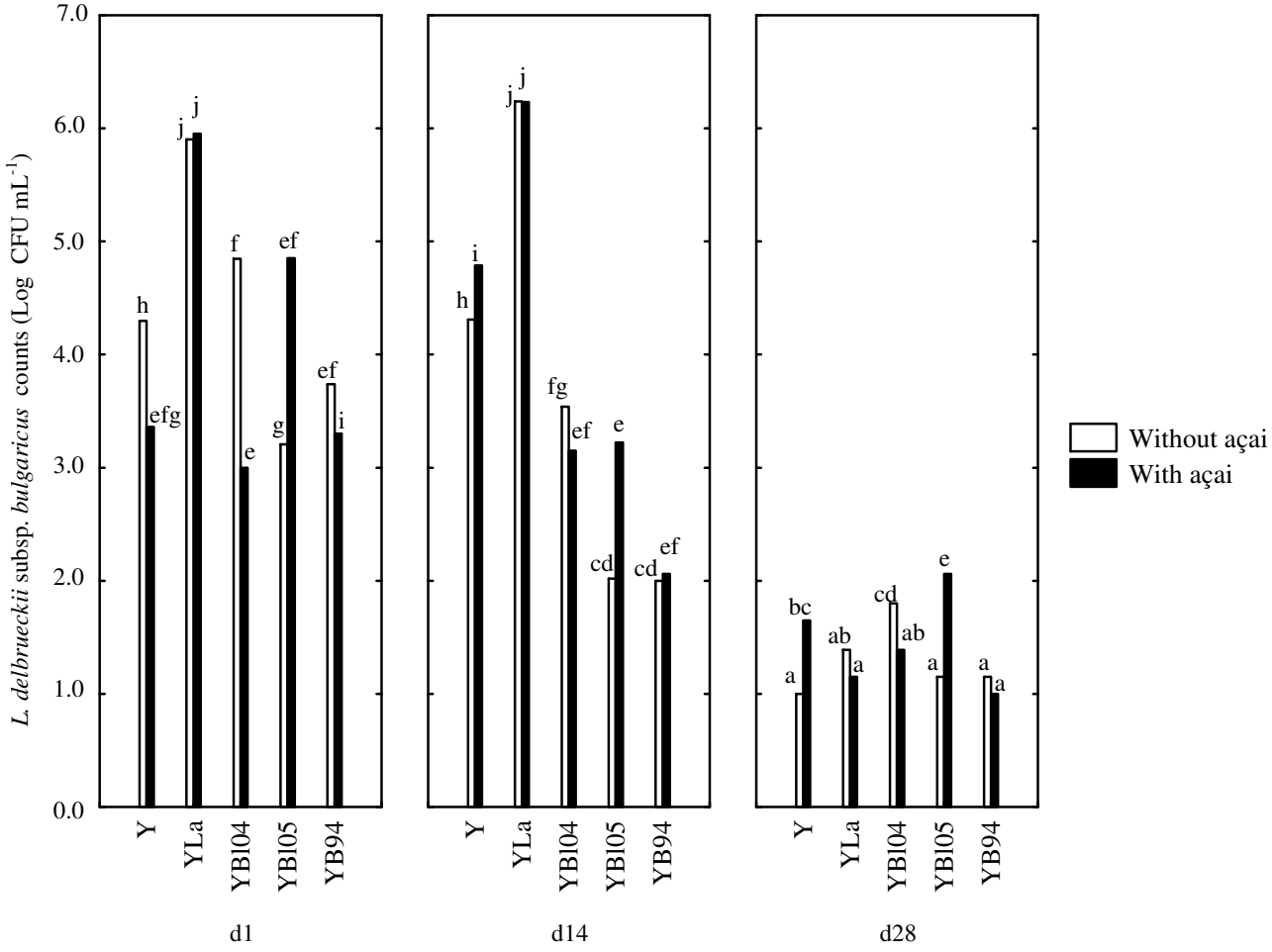

Figure 2.3. *Lactobacillus delbrueckii* subsp. *bulgaricus* counts in yogurt with and without açai pulp during shelf-life. Abbreviations: Yogurts associated with *L. acidophilus* L10 (YLa), *B. lactis* Bl04 (YBl04), *B. longum* Bl05 (YBl05) and *B. lactis* B94 (YB94). Means with different letters are significantly ($P < 0.05$) different. N = 40.

Probiotic counts in yogurt, with or without açai pulp, over 28 days of cold storage are shown in Figure 2.4. On day 1, the presence of açai pulp had no significant effect ($P > 0.05$) on the growth of *B. lactis* Bl04 and B94 strains, however, the presence of açai had a negative effect ($P < 0.05$) on the growth of *B. longum* Bl05 compared to the control without fruit (Figure 2.4). Furthermore, on day 1 it was observed that *B. lactis* (Bl04) counts in yogurts, with or without açai, were significantly higher ($P < 0.05$) than any other probiotic strains (Figure 2.4).

On day 14, the *L. acidophilus* L10 strain in açai yogurt showed lower counts ($P < 0.05$) compared to the control without pulp, pointing to a change in the L10 strain behavior in relation to the 1st day (Figure 2.4). In the second week of cold storage, the probiotics *B. lactis* Bl04, *B. lactis* B94 and *B. longum* Bl05 in açai yogurt had higher counts ($P < 0.05$) compared to their respective controls without fruit (Figure 2.4). Although on day 14 *B. lactis* Bl04 and B94 had counts ~8 Log CFU mL^{-1}, *B. longum* Bl05 showed counts ~6 Log CFU mL^{-1}, representing a better adaptation to the product conditions of *B. lactis* strains compared to *B. longum* (Figure 2.4). The higher count of 1 Log CFU mL^{-1} for B94 in açai yogurt compared to its control without açai was remarkable after two weeks of cold storage (Figure 2.4). This observation has not been reported before, indicating a synergistic effect between the fruit pulp and the *B. lactis* B94 strain at 14 days of cold storage (Figure 2.4).

After 28 days, the enumeration of *L. acidophilus* L10, *B. lactis* Bl04 and *B. longum* Bl05 was higher ($P < 0.05$) in açai yogurt, i.e., 7.65, 9.36, 5.42 Log CFU mL^{-1}, respectively (Figure 2.4). Between days 14 and 28, there were significant reductions ($P < 0.05$) in the *B. lactis* B94 and *B. longum* Bl05 counts in yogurts, with or without açai (Figure 2.4). The last enumeration at day 28 pointed to a lack of influence ($P > 0.05$) of açai pulp upon B94 viability. This observation indicates a probable change of synergistic behavior previously observed at 14 days of storage.

A strong variability in stability between species and strains of probiotics bacteria in fruit juices was pointed out by Champagne and Gardner (2008). Celik and Ibakirci (2003) observed that the mean count of LAB of mulberry yogurts was significantly lower than for the control. The same observation was reported by Ozturk and Oner (1999) about yogurts fermented with concentrated grape juice. However, in this study açai pulp showed no negative effect on probiotic bacteria counts after 4 weeks of cold storage (Figure 2.4). In a previous study, Almeida *et al.* (2008) reported the counts of *L. acidophilus* and *B. bifidum* (both Chr. Hansen, Valinhos, Brazil) at ~8 Log CFU mL^{-1} and 7 Log CFU mL^{-1} respectively, at day 21 of cold storage of yogurt containing açai pulp. Similar counts of *L. acidophilus* L10 strain were observed in the present study at day 28 of storage at 4ºC (7.6 Log CFU mL^{-1}) whereas *B. animalis* ssp. *lactis* Bl04 showed counts of 9.4 Log CFU mL^{-1} at day 28 of cold storage, indicating improved microorganism viability compared to *B. bifidum*, as

previously observed by Almeida *et al*. (2008). Vasiljevic and Shah (2008) reported that the recommended level of viable probiotic bacteria ranges from 6 – 8 Log CFU mL^{-1} at the end of cold storage, and these counts were reached by *L. acidophilus* L10 and *B. lactis* Bl04. However, *B. longum* Bl05 and *B. lactis* B94 didn't show satisfactory counts at day 28 of storage, indicating that an increase in the initial counts of the inoculum may be required.

The counts of *S. thermophilus* were not correlated with *L. acidophilus* L10 counts but showed a moderate positive correlation with *B. lactis* counts (r = 0.5107; Figures 2.2 and 2.4). Moreover, the pH showed a moderate correlation (r = 0.5519) with the count of probiotic microorganisms. The relationship between pH and probiotic bacteria viability in the presence of açai pulp has not been reported before, however our results are in accordance with the observations of Kailasapathy et al. (2008) who pointed to a higher *L. acidophilus* L10 viability in yogurt with pH

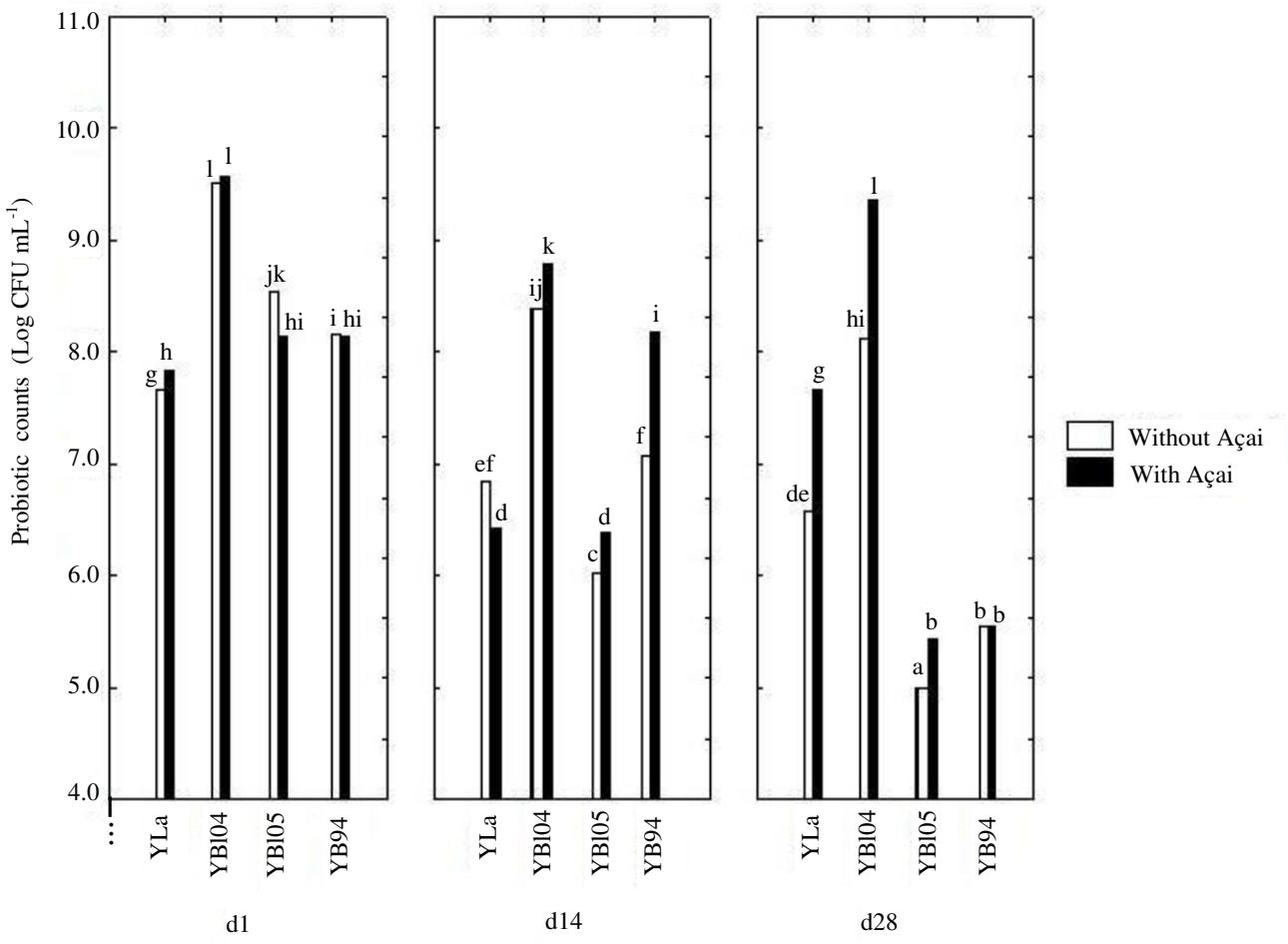

Figure 2.4. Probiotic counts in yogurt with and without açai pulp during shelf-life.

Abbreviations: Yogurts associated with *L. acidophilus* L10 (YLa), *B. lactis* Bl04 (YBl04), *B. longum* Bl05 (YBl05) and *B. lactis* B94 (YB94). Means with different letters are significantly ($P < 0.05$) different. N = 8.

between 4.1 and 4.5, during cold storage. Our data are also in agreement with Donkor *et al*. (2006) who pointed to greater *L. acidophilus* L10 and *L. paracasei* L26 viability in yogurt, compared to *B. animalis* ssp. *lactis* B94, after 28 days of cold storage. The higher counts of *L. acidophilus* L10 and *B. lactis* Bl04 can also be due to the higher residual lactose level of açai yogurts containing these probiotic strains (data not shown). A possible explanation for this observation could be the preference of *L. acidophilus* in the metabolization of sucrose which is present in açai pulp, rather than lactose (NIELSEN & GILLILAND, 1992).

2.3.4. Fatty acid profile

The chromatograms of açai pulp presented the following fatty acid profile in g per 100 g^{-1} of lipids (mean ± standard deviation): 0.50±0.21 (C4:0), 0.47±0.25 (C6:0), 0.62±0.23 (C10:0), 23.91±1.55 (C16:0), 3.98±0.32 (C16:1), 1.89±0.06 (C18:0), 10.89±0,46 (C18:2) and 0.62±0,03 (C18:3). These values are in agreement with Nascimento *et al.* (2008) who report the use of açai pulp as an essential fatty acid source.

During the fermentation of milk, fatty acid profiles progressively change as a result of microbial growth (EKINCI *et al.,* 2008). The production of free fatty acids by probiotic LAB through lipolysis of milk fat has been reported by Coskun and Ondul (2004) and Yadav *et al.* (2007). In the human large intestine, short-chain fatty acids (SCFA) are formed by anaerobic bacterial metabolism from carbohydrates that are not absorbed by the cells of the intestinal wall (MACFARLANE & MACFARLANE, 2003).

The content of SCFA (C4:0 and C6:0) was significantly shorter ($P < 0.05$) in açai yogurts with *B. lactis* B94 or *L. acidophilus* L10 strains compared to their controls without fruit. However, there was a reduction ($P < 0.05$) in medium-chain fatty acids (MCFA; C8:0 to C15:1) content in açai yogurt with B94 strain compared to its control without pulp (Figure 2.5). Açai increased ($P < 0.05$) the long-chain fatty acid (LCFA) content in yogurts with *B. lactis* B94, *B. lactis* Bl04 or *L. acidophilus* L10 strains compared to their controls without fruit (Figure 2.5). In yogurts without açai, it was observed that *B. lactis* B94 produced higher amounts ($P < 0.05$) of SCFA and MCFA, and lower amounts of LCFA than *B. lactis* Bl04, pointing to a strain dependent effect on fatty acid profile in this case (Figure 2.5).

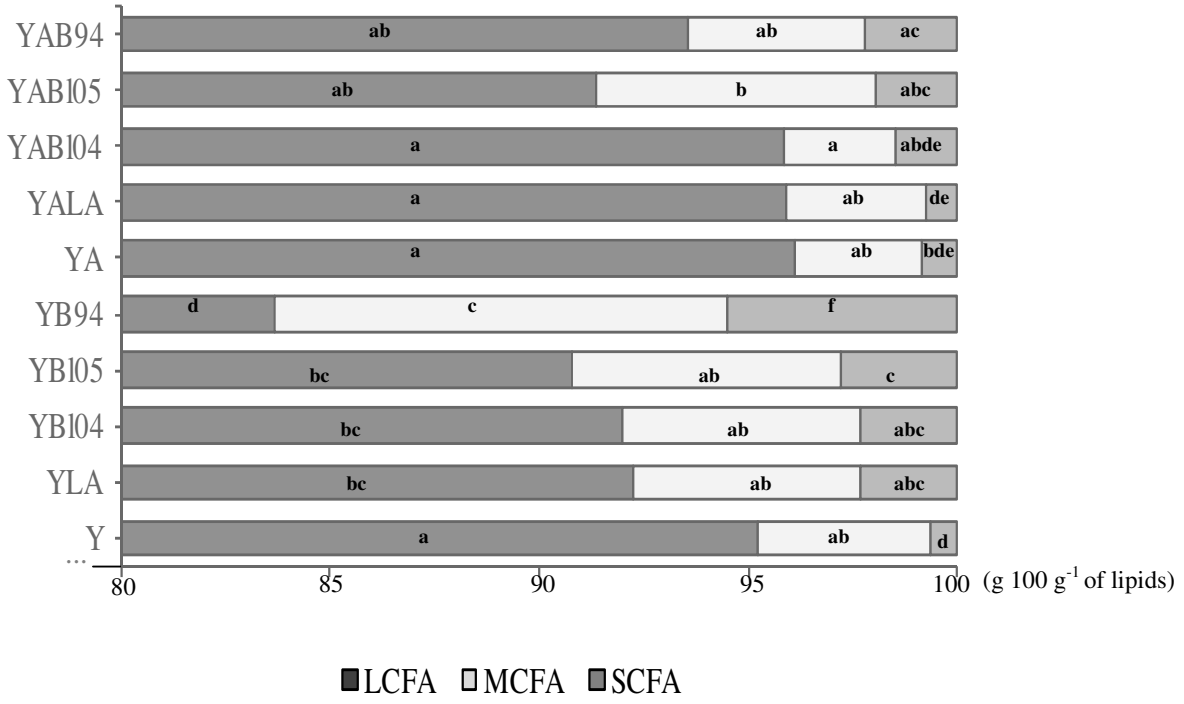

Figure 2.5. Açai (YA) and control (Y) yogurts fatty acid profile (g $100g^{-1}$of lipids) according to carbon chain length, observed at the day after fermentation (day 1). Means with different letters to the same carbon chain length are significantly (P< 0.05) different. N = 12.

Abbreviations: short chain fatty acid (SCFA, C4:0 to C6:0); medium chain fatty acid (MCFA, C8:0 to C15:1); long chain fatty acid (LCFA, C16:0 to C18:3). Yogurts associated with *L. acidophilus* L10 (YLa; YALa), *B. lactis* Bl04 (YBl04; YABl04), *B. longum* Bl05 (YBl05; YABl05) and *B. lactis* B94 (YB94; YAB94).

All açai yogurts showed higher content (P < 0.05) of monounsaturated fatty acids (MUFA) and PUFA compared to their controls without pulp (Table 2.3). On the other hand, yogurts without açai showed the highest content (P < 0.05) of saturated fatty acids (SFA; Table 2.3). Amongst yogurts without fruit, *B. lactis* B94 increased SFA and reduced significantly (P < 0.05) MUFA contents. Nevertheless, among açai yogurts those with *B. longum* Bl05 showed the higher SFA content and the lower MUFA and PUFA contents (P < 0.05; Table 2.3). These observations suggest a strain dependent effect upon unsaturated fatty acid profile of yogurts.

Polyunsaturated fatty acids, ω-3 and ω-6 have been used as dietary supplements and are reported to positively affect the immune system (BOMBA *et al*., 2006). Besides that, PUFAs

promote adhesion of probiotics to mucosal surfaces and alleviate changes related to allergic inflammation (KANKAANPÄA *et al*., 2001; DAS, 2002).

Palmitic acid (C16:0) content was significantly lower ($P < 0.05$) in açai yogurts with *B. animalis* ssp. *lactis* Bl04 and B94 strains and without probiotic (YA) compared to their respective controls without fruit (Y). However, açai pulp contributed significantly to the increase ($P < 0.05$) in the palmitoleic acid (C16:1) content in yogurts compared to controls without pulp (Table 2.3).

Stearic acid (C18:0) content was approximately twice as high amongst control yogurts compared to açai yogurts. Moreover, açai yogurts showed higher ($P < 0.05$) contents of oleic (C18:1), linoleic (C18:2) and α-linolenic (ALA; 18:3) acids compared to control yogurts without fruit (Table 2.3). It is important to emphasize that according to Zhao *et al.* (2007) a higher dietetic intake of ALA promotes reduction in lipids, lipoproteins and in the inflammatory markers C-reactive protein and cell adhesion molecules, which leads to a decreased risk of cardiovascular disease. So, based on the results of the present study the consumption of açai yogurts can contribute to an increase of ALA dietary intake.

Table 2.3. Fatty acid profile (g $100g^{-1}$) of yogurts without (Y) and with (YA) açai pulp associated with different probiotic strains.

Yogurts	SFA	MUFA	PUFA	16:0	16:1	18:0	18:1	18:2	CLA	ALA
Without açai										
Y	65.77±0.11 ^{b}	26.89±0.09^{a}	3.73±0.05^{a}	32.46±0.17^{bc}	1.75±0.01^{a}	13.36±0.11 ^{b}	23.83±0.08 ^{a}	2.01±0.02 ^{a}	1.19 ±0.03 ^{bcd}	0.53 ±0.01 ^{b}
YLa	66.86±0.36 ^{b}	26.13±0.26^{a}	3.57±0.06^{a}	31.41±0.21^{bcd}	1.69±0.02^{a}	13.10±0.19 ^{b}	23.18±0.29 ^{a}	1.90 ± 0.03 ^{a}	1.16 ±0.01 ^{abcd}	0.51 ±0.02 ^{b}
YBl04	67.08± 0.41^{b}	25.83±0.37^{a}	3.60±0.01^{a}	31.54±0.29^{bcd}	1.67±0.03^{a}	12.83±0.24 ^{b}	22.88±0.40 ^{a}	1.93± 0.01 ^{a}	1.17 ±0.03 ^{abcd}	0.51± 0.03 ^{b}
YBl05	68.93±0.33^{b}	24.56±0.23^{a}	3.25±0.07^{ac}	31.73±0.58^{bc}	1.71±0.05^{a}	11.95±0.50 ^{b}	21.52±0.69 ^{a}	1.78± 0.05 ^{a}	1.05± 0.03 ^{abe}	0.42± 0.05 ^{b}
YB94	91.59±0.84^{d}	6.24±0.56^{c}	2.31±0.59^{c}	33.27±1.67^{c}	1.80±0.03^{a}	19.15±0.50 ^{c}	2.89± 0.89 ^{c}	1.58 ±0.48 ^{a}	0.88 ±0.06 ^{e}	0.73± 0.02 ^{cd}
With açai										
YA	44.35±2.53^{a}	44.24±2.17^{b}	9.24±0.87^{b}	27.20±1.91^{a}	3.11±0.30^{bc}	5.92 ±0.71 ^{a}	40.54±3.52 ^{b}	7.19 ±0.61 ^{c}	1.16 ±0.08 ^{abcd}	0.88± 0.18 ^{a}
YALa	45.55±2.60^{a}	43.39±1.70^{b}	8.90±0.54^{b}	28.10±1.33^{ad}	3.26±0.19^{b}	5.64± 0.37 ^{a}	39.52±1.94 ^{b}	7.03 ±0.38 ^{bc}	1.02 ±0.05 ^{abe}	0.85 ±0.11 ^{a}
YABl04	45.31±2.10^{a}	43.62±1.61^{b}	9.03±0.40^{b}	27.12±0.57^{a}	2.83±0.07^{cd}	6.87 ±0.24 ^{a}	40.18±1.26 ^{b}	6.77± 0.22 ^{bc}	1.41± 0.04 ^{f}	0.85 ±0.09 ^{a}
YABl05	55.75±3.04^{c}	36.72±2.08^{d}	7.21±0.53^{d}	29.42±0.44^{abd}	3.08±0.10^{b}	5.11± 0.51 ^{a}	32.82±2.79 ^{d}	5.54± 0.57 ^{d}	0.96± 0.03 ^{ae}	0.71± 0.10 ^{d}
YAB94	49.30±2.46^{a}	40.87±1.61^{b}	8.45±0.30^{b}	27.54±0.85^{a}	2.65±0.10^{d}	6.65 ±0.48 ^{a}	37.53±2.32 ^{b}	6.27± 0.37 ^{d}	1.36± 0.04 ^{df}	0.82± 0.06 ^{a}

Means (N = 12) ± standard deviation with different letters in the same column are significantly different ($P < 0.05$).

Abbreviations: YLa and YALa yogurts associated with *L. acidophilus* L10; YBl04 and YABl04 yogurts associated with *B. lactis* Bl04; YBl05 and YABl05 yogurts associated with *B. longum* Bl05; YB94 and YAB94 yogurts associated with *B. lactis* B94. SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid. 16:0, Palmitic acid; 16:1, Palmitoleic acid; 18:0, Stearic acid; 18:1, Oleic acid; 18:2, Linoleic acid; CLA, Conjugated linoleic acid (c9t11-18:2) ; ALA, α-Linolenic acid, 18:3.

The CLA (c9t11-18:2) content ranged from 0.88 to 1.19 g 100 g^{-1} in control yogurts, and from 0.96 to 1.41 g 100 g^{-1} in açai yogurts (Table 2.3). The presence of açai pulp stimulated ($P < 0.05$) the CLA increase in yogurt with *B. lactis* Bl04 - around 17% higher than in yogurt without pulp (Table 2.3). Moreover, açai yogurt with *B. lactis* B94 showed a 35% increase in CLA levels compared to yogurts without pulp, indicating a synergistic effect of this probiotic strain and açai pulp (Table 2.3). Sieber *et al.* (2004) reported that the presence of fructose increases production of c9t11-18:2 by *L. delbrueckii* ssp. *lactis* 12586. The same might have happened in the case of açai yogurts with *B. lactis* Bl04 and B94 in the present study since açai pulp is a source of fructose (SCHAUSS *et al.*, 2006). On the other hand, CLA is an intermediate in the biohydrogenation of linoleic acid (18:2) to stearic acid by LAB (OGAWA *et al.*, 2005). Since açai pulp is a source of linoleic acid, the açai pulp may have stimulated CLA production by *B. lactis* strains. This observation has not been reported before.

2.4. Conclusions

The addition of açai pulp into milk favored an increase in *L. acidophilus* L10, *B. animalis* ssp. *lactis* Bl04 and *B. longum* Bl05 counts at the end of 28 days of cold storage. Yogurts without açai pulp showed a greater content of saturated fatty acids whereas açai yogurts showed a higher content of polyunsaturated fatty acids. This study demonstrated that the production of bioactive lipid components such as α-linolenic and conjugated linoleic acids can be enhanced by açai pulp addition during fermentation of skim milk prepared with *B. animalis* ssp. *lactis* Bl04 and B94 strains, offering potential health benefits of this probiotic açai yogurt.

CHAPTER III

FIBERS FROM FRUIT BY-PRODUCTS ENHANCE PROBIOTIC VIABILITY AND FATTY ACIDS PROFILE AND INCREASE CLA CONTENT IN YOGHURTS

3.1. Introduction

The waste of food is an unlucky reality worldwide (FAO/WHO, 2008). In particular, during the processing of fruit for pulp production, around 65-70% by weight of the raw material is lost, leading to serious environmental problems (LOUSADA JR *et al*., 2006). However, it was demonstrated that some fibers of fruit by-products show functional properties such as water-holding, swelling, gel forming, bile acid binding, and cation-exchange capacities (LAMSAL & FAUBION, 2009). Among the promising fruit by-products are the peels of apple, banana and passion fruit, mainly because of their content of insoluble and soluble dietary fibers (DF), pectin and fructooligosaccharides. These prebiotics are in fact able to selectively stimulate the growth and activity of the gut microbiota, particularly lactobacilli and bifidobacteria (DAVIS & MILNER, 2009).

It was reported that 100 g of dry apple (*Malus* sp., Rosaceae) by-product contain around 46 g of insoluble fiber, 14 g of soluble fiber (CHEN *et al*., 1988), while pectin and fructooligosaccharide account for 8-12 and 4.9 % of total dietary fiber (DF), respectively NAWIRSKA & KWASNIEWSKA, 2005). On the other hand, banana (*Musa* sp., Musaceae) by-product contains around 43-49 g of total DF, 1 g of inulin, 6 g of fructooligosaccharide and 10-20 g of pectin per 100 g of dry matter, in addition to significant amounts of α-linolenic acid (ALA), essential amino acids and micronutrients such as Mg, K, P and Ca (EMAGA *et al*., 2007; EMAGA *et al*., 2008; MOHAPATRA *et al*., 2010). Finally, the rind of yellow passion fruit (*Passiflora edulis* var. *flavicarpa* Degenerer, Passifloraceae) is composed of approximately 60 g of total insoluble dietary fiber and 20 g of pectin per 100 g of dry matter (YAPO & KOFFI, 2008; SALGADO *et al*., 2010). The degrees of polymerization of homogalacturonan in apple and banana peels and in yellow passion fruit rind were found to be approximately 78, 67 and 93%, respectively (RENARD *et al*., 1995; YAPO, 2009).

The dietary intake of fibers and probiotics exert a positive impact on the development of the intestinal microbiota and are reported to relieve constipation and reduce the incidence of colon

cancer (FARNWORTH, 2008). Moreover, epidemiological investigations relate the incidence of civilization-induced diseases to insufficient DF ingestion from fruit and vegetables (NAWIRSKA & KWASNIEWSKA, 2005). Finally, the beneficial effects on probiotics viability exerted by some ingredients such as fruit pieces or pulp to dairy foods have been reported (KOURKOUTAS *et al*., 2006; SENDRA *et al.*, 2008; ESPÍRITO SANTO *et al*., 2010).

Some strains of bacteria are able to change the fatty acids profile of milk and produce functional fatty acids during the fermentation as the result of their growth and metabolism (COSKUN & ONDUL, 2004; YADAV *et al.*, 2007; EKINCI *et al*., 2008). Among the functional fatty acids present in the milk, the conjugated linoleic acids (CLA) stand out. It was observed that long chain polyunsaturated fatty acids such as ALA and CLA not only promote the adhesion of some *Lactobacillus* species to the mucosal surface of the gut, but also alleviate the symptoms associated to bowel inflammation (KANKAANPÄA *et al*., 2001; DAS & FAMS, 2002). CLA present in the milk is produced as an intermediate compound in the biohydrogenation of the linoleic acid to stearic acid by the metabolism of bacteria in the rumen (KIM & LIU, 2002). Furthermore, Oh *et al.* (2003) and Ogawa *et al* (2005) reported that more than 250 bacterial strains from 14 genera such as *Enterococcus, Pediococcus, Propionibacterium, Lactobacillus* and *Bifidobacterium* were found to produce CLA from linoleic acid.

Previous studies of our research group showed that the level of CLA can be increased by the addition of some ingredients to the milk to be fermented (OLIVEIRA *et al*., 2009; ESPÍRITO SANTO *et al*., 2010). Oliveira *et al*. (2009) employed polysaccharides such as maltodextrin, oligofructose and polydextrose and evaluated their influence on CLA content in milks fermented by *Streptococcus thermophilus* and one strain of *Lactobacillus* or *Bifidobacterium animalis* subsp. *lactis*. Those commercially available prebiotics are far chemically simpler ingredients than total dietary fruit fibers, such as those from apples, bananas and passion fruit, which contain soluble and insoluble fibers, others carbohydrates, phenolic compounds, proteins, minerals and vitamins (NAWIRSKA & KWASNIEWSKA, 2005; MOHAPATRA *et al*., 2010 and SALGADO *et al*., 2010). To improve the fatty acids profile of the yoghurts fermented by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and *B. lactis* Bl04 or B94 strains, Espírito Santo *et al.* (2010) added açai pulp to the milk thus offering an extra amount of polyunsaturated fatty acids and CLA precursors. Similarly, Xu *et al.* (2005) demonstrated that the addition of 0.1% linoleic acid is able to increase significantly the CLA content in yoghurts fermented by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in co-culture with some probiotic strains, but not in yoghurts fermented by the starter culture alone.

The addition of a complex ingredient such as fruit pulp or total dietetic fruit fiber in the formulation of a probiotic product is a challenge, mainly due to the presence of antimicrobial compounds in the fruit and its acidity (ESPÍRITO SANTO *et al*., 2011). The trend to use by-products from fruits can contribute to obtain cheap, high value-added dairy products able to increase the daily intake of dietary fiber and improve the yoghurt bacteria viability (FERNANDEZ-GARCIA & MCGREGOR, 1997; STAFFOLO *et al*., 2004; SENDRA *et al*., 2008). But, what is the effect of the total dietetic fruit fiber on the fatty acids profile of probiotic yoghurts? This question is still unanswered.

To contribute to fulfill this gap, the aim of this study was to evaluate the effect of the addition of the total dietetic fiber from apple, banana and passion fruit processing by-products on the fatty acids profile and counts of viable microorganisms in yoghurts co-fermented by a starter culture plus one of four different probiotic strains. Post-acidification and titratable acidity of the yoghurts were also evaluated during cold storage. The main novelty of such an approach should be recognized in its attempt to reveal in probiotic yoghurts the transformation of fatty acids profile, particularly CLA, not through the usual addition of CLA precursors or commercial soluble fibers, but through the addition of cheap total dietary fibers, by-producst of the fruit pulp industry. Compared with previous study made with açai pulp as a lipid source (ESPÍRITO SANTO *et al.,* 2010), an additional innovating issue of this work is the use of the above three different fruit fibers to increase the viability of probiotics and to produce yoghurts with higher dietary fiber content.

3.2. Materials and methods

3.2.1. Preparation of fibers

Apple (gala cultivar), banana (cavendish cultivar) and passion fruit (yellow cultivar) by-products were obtained from a factory of fruit pulp located in the city of Jundiai, São Paulo State, Brazil. In the factory the fruits were cleaned and pressed in a hydraulic presser at room temperature (25 °C). The by-products were collected just after the industrial processing and frozen at -25 °C. They were transported to the laboratory as frozen material in polystyrene container the next day and stored in a bench-scale freezer, in order to avoid the attack of the organic material by contaminating microorganisms. In the next day, the peels of apple, banana and passion fruit were thawed, cleaned under running tap water and decontaminated in 5 ppm of chlorine active hypochlorite solution for 30 min. The material was then dried in oven with circulating air at 60 °C until constant weight. The dry peels were reduced to fine powders in a Bimby processor, model TM 31 (Vorwerk, Wuppertal,

Germany). To make the mixture of the fiber powder with the reconstituted milk easier, the particle size was standardized to less than 17.7 μm and measured through sieves (Granutest, São Paulo, Brazil) with mesh diameters of 200, 119, 59, 42 and 17.7 μm. The resulting fiber powders (Figure 3.1) were stored in sealed glass pots and kept under refrigeration at 4°C.

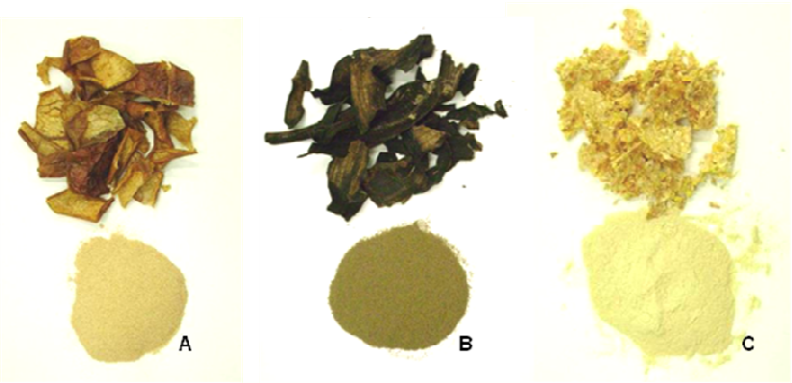

Figure 3.1: Fiber powders of apple (A), banana (B) and passion fruit (C) by-products

3.2.2. Yoghurt manufacture

Skim milk powder (2 g fat/L; Molico, Nestlé, Araçatuba, Brazil) was reconstituted to 12% (w/w) in distilled water and divided into two milk bases: the one containing 1% fiber and the other without fiber (control). The milk bases were then treated thermally as described by Espírito Santo *et al.* (2010).

The freeze-dried starter yoghurt culture CY340 (DSM, Moorebank, NSW, Australia) was composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. It was combined with one of four probiotic cultures, specifically *Lactobacillus acidophilus* L10 (DSM), *Bifidobacterium animalis* subsp. *lactis* Bl04 and HN019 (Danisco, Madison, WI, USA), or B94 (DSM). The inocula preparation and the inoculation of milk bases were done accordingly to Espírito Santo *et al*. (2010). In order to make comparisons, the strains chosen for this study were the same previously investigated by our group in the preparation of others dairy fermented products (OLIVEIRA *et al.,* 2009; ESPIRITO SANTO *et al*, 2010). Because in one of our previous studies the same starter co-culture (CY340) showed no pronounced effect on fatty acid profile in yoghurts (ESPIRITO SANTO *et al*, 2010), it was always used in combination with a probiotic strain, instead of alone.

Sixteen different yoghurts were prepared combining 3 types of fruit fibers (plus control without fiber) with only one of the 4 probiotic strains. Two independent fermentations were performed on different days and replicated twice. After inoculation, the fermentations were carried out at 42 °C in a CINAC system (Cynetique d´acidification, Ysebaert, Frépillon, France) as described by Spinnler and Corrieu (1989). When the desired pH was reached (pH 4.5) the flasks with the yoghurts were cooled to 15 °C in ice bath, and then the clot was broken. Afterwards, the fermented milk was packed and sealed in 50 mL polypropylene pots and cooled to 4°C. All sampling and analyses were performed after 1 day (d 1), 14 days (d 14) and 28 days (d 28) of cold storage at this temperature.

3.2.3. Determination of chemical composition of fruit fibers and heat treated milk bases

Moisture content was determined gravimetrically (70 ± 1°C for 6 h in a vacuum oven) using 4-5 g of sample. Lipids, protein and ash were analyzed according to AACC (2000) methods 30-25, 46-13 and 08-01, respectively. Total dietary fiber was determined using the 985.29 AOAC (1999) method. The measurements were done in triplicate.

3.2.4. Post-acidification and titratable acidity

Four pots of each treatment were taken and homogenized before analyses. The yoghurt post-acidification was determined as pH after 1, 14 and 28 days using a pH meter, model Q-400M1 (Quimis, São Paulo, Brazil). The titratable acidity was analyzed according to recommendations of AOAC (1995). The results were expressed as the means of four replicates.

3.2.5. Microbiological analyses

Bacterial counts of each treatment were carried out in quadruplicate after 1, 14 and 28 days. Samples (1 mL) were diluted with 0.1 % sterile peptonated water (9 mL). Afterwards, serial dilutions were carried out, and bacteria were counted, applying the pour plate technique (KODAKA *et al*., 2005). All media were obtained from Oxoid (Basingstoke, UK). In co-cultures, *S. thermophilus* colonies were enumerated in M17 agar, whereas those of *L. delbrueckii* subsp. *bulgaricus* were counted in MRS agar (pH 5.4), both under aerobic incubation at 37 °C for 48 h. Enumerations of *L. acidophilus* were carried out in MRS agar (pH 6.2) plus 10 μL mL^{-1} clindamycin (Sigma, St. Louis, MO, USA), and those of *B. lactis* B04, B94 and HN019 in

Reinforced Clostridial Agar plus 100 μL mL^{-1} of dicloxacillin (Sigma). The media M17 and MRS agar (pH 5.4) were prepared according to Jordano et al. (1992) and Dave and Shah (1996). The media MRS agar (pH 6.2) plus clindamycin was prepared as described by Tharmaraj and Shah (2003) and Saccaro *et al*. (2009). All probiotic microorganisms were incubated under anaerobic conditions, provided by AnaeroGen (Oxoid), at 37 °C for 72 h. Cell concentration was expressed as Log CFU mL^{-1} of yoghurt.

3.2.6. Fatty acid profiles

The fatty acid profile of yoghurts was determined 1, 14 and 28 days after cold storage according to the method of AOCS (1997). Four pots of each type of yoghurt were taken and homogenized before the cold extraction of the lipid fraction. The result of each treatment was reported as mean value of twelve runs.

3.2.7. Statistical analyses

The Statistica Software 8.0 (Statsoft, Tulsa, OK, USA) was used in all statistical analyses. The data were analyzed by two way ANOVA using Tukey test at $P < 0.05$ to compare mean values, and the General Linear Model was applied to evaluate the effect of time and different interactions such as probiotic culture and fiber type. Statistically significant differences were indicated by labeling the mean values with different letters.

3.3. Results and Discussion

3.3.1. Chemical composition of fruit fibers and milk bases

The chemical composition of the total dietary fibers and the heat treated milk bases are shown in Table 3.1. In general, the chemical composition of apple, banana and passion fruit fibers are in accordance with the results of Nawirska and Kwasniewska (2005), Mohapatra *et al*. (2010) and Salgado *et al.* (2010), respectively. As expected, the addition of fiber increased by 1 g/100 g the total solids (total weight – moisture) of the heat treated milk bases.

Table 3.1. Chemical composition in g/100 g of the total dietetic fibers and the heat treated skim milk bases.

	Total dietetic fibers			Skim milk bases			
	Apple	Banana	Passion fruit	Control	Apple	Banana	Passion fruit
Proteins	5.21±0.02	10.01±0.04	6.32±0.01	6.14±0.02	6.19±0.02	6.29±0.02	6.20±0.02
Lipids	5.03±0.01	8.15±0.02	6.96±0.01	0.20±0.00	0.25±0.01	0.29±0.01	0.27±0.01
Carbohydrates	78.01±0.88	63.06±0.69	74.33±0.75	6.34±0.02	7.16±0.03	6.87±0.02	7.02±0.02
Ash	2.17±0.01	10.59±0.02	3.88±0.02	0.70±0.01	0.72±0.01	0.81±0.01	0.74±0.01
Moisture	9.05±0.46	8.12±0.37	9.40±0.44	86.57±0.55	85.68±0.61	85.47±0.59	85.27±0.47
Total dietary fiber	63.27±0.51	51.19±0.43	65.08±0.56	0.00±0.00	0.61±0.02	0.52±0.00	0.64±0.01

$N = 3$.

3.3.2. Post-acidification and total titratable acidity

The addition of fruit fiber promoted a statistically significant ($P < 0.05$; $N = 64$) reduction in the initial pH of all heat-treated skim milk bases, specifically from 6.53 in control milk to 6.42 in milks supplemented with apple and banana fibers and to 6.30 in that supplemented with passion fruit fiber (results not shown).

After 1 day of cold storage, only the passion fruit fiber yoghurt co-fermented by *Lactobacillus acidophilus* L10 showed a pH significantly lower ($P < 0.05$) than its control without fiber (Table 3.2), whereas no statistically significant effect ($P > 0.05$) on post-acidification could be ascribed to the other fiber-probiotic combinations. The fibers of banana and passion fruit significantly increased ($P < 0.05$) the titratable acidity of yoghurt co-fermented by *L. acidophilus* L10 compared to the control. The same was observed with all the fibers using *Bifidobacterium animalis* subsp. *lactis* HN019 and only with the banana fiber using *B. animalis* subsp. *lactis* Bl04.

At the end of four weeks of shelf life (28 days), the post-acidification exhibited almost the same results as those of d 14 ($P < 0.05$) in Table 3.2. A slight increase of pH ($P < 0.05$) was observed in yoghurts supplemented with apple and banana fibers and co-fermented by *B. animalis* subsp. *lactis* Bl04 compared to the controls. Similarly, Espírito Santo *et al.* (2010) reported that the pH of açai yoghurts fermented by the Bl04 strain was significantly higher ($P < 0.05$) than that of their respective controls without fruit, which indicates that the Bl04 strain in the presence of some fruity products may reduce its organic acid production. Regarding the control and passion fruit fiber yoghurts, it is noteworthy that the higher pH shown in Bl04 fermented apple and banana fibers

yoghurts at 1 day is positively correlated (r = 0.855) with the high counts of this bacterium, which indicates a possible synergism between fruit fiber and probiotic via acidity regulation. On the other hand, no significant differences in pH due to fiber addition ($P > 0.05$) were observed in the present study in yoghurts co-fermented by the other probiotics.

Table 3.2. Post-acidification (pH) and titratable acidity of yoghurts with and without fibers during 4 weeks of cold storage.

	pH			Titratable acidity (% lactic acid)		
Yoghurt	d 1	d 14	d 28	d 1	d 14	d 28
CL10	4.42^{c}	4.29^{ef}	4.30^{de}	0.81^{ab}	0.94^{cd}	0.99^{a}
AL10	4.40^{abc}	4.31^{ef}	4.31^{bcd}	0.82^{abc}	0.99^{efg}	1.06^{abcd}
BL10	4.39^{abc}	4.33^{f}	4.34^{e}	0.86^{cd}	0.91^{abcd}	1.04^{abcd}
PL10	4.36^{a}	4.31^{ef}	4.32^{cde}	0.86^{cd}	0.88^{a}	0.99^{a}
CBl04	4.44^{abc}	4.22^{a}	4.23^{a}	0.86^{cd}	0.91^{abcd}	1.02^{abc}
ABl04	4.44^{bc}	4.28^{de}	4.29^{bcd}	0.93^{e}	0.96^{de}	1.05^{abcd}
BBl04	4.41^{abc}	4.27^{de}	4.29^{cde}	1.04^{f}	0.99^{ef}	1.11^{cd}
PBl04	4.41^{abc}	4.24^{ab}	4.25^{bc}	0.96^{e}	0.91^{abcd}	1.07^{abcd}
CHN019	4.43^{abc}	4.25^{ab}	4.25^{ab}	0.87^{d}	0.88^{ab}	1.01^{ab}
AHN019	4.41^{bc}	4.28^{bcd}	4.27^{bcd}	0.93^{e}	0.94^{cd}	1.07^{abcd}
BHN019	4.38^{abc}	4.27^{bcd}	4.25^{bcd}	0.95^{e}	1.03^{fg}	1.04^{abcd}
PHN019	4.40^{ab}	4.25^{bc}	4.25^{ab}	0.97^{e}	1.04^{g}	1.12^{d}
CB94	4.43^{abc}	4.25^{bc}	4.25^{abc}	0.82^{abc}	0.90^{abc}	1.04^{abcd}
AB94	4.40^{abc}	4.28^{bc}	4.28^{abc}	0.85^{bcd}	0.90^{abc}	1.03^{abc}
BB94	4.38^{abc}	4.27^{bc}	4.28^{bcde}	0.78^{a}	0.93^{bcd}	1.07^{abcd}
PB94	4.42^{abc}	4.28^{ab}	4.29^{ab}	0.85^{bcd}	0.93^{bcd}	1.09^{bcd}

Means with different letters in the same column are significantly different ($P < 0.05$), $N = 64$. Standard deviations were lower than 0.05 and are not shown.

Abbreviations: d1, d14 and d28 mean shelf-lives of 1, 14 and 28 days. Yoghurts co-fermented by *L. acidophilus* L10: control (CLa), with apple fiber (ALa), with banana fiber (BLa), with passion fruit fiber (PLa). Yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04: control (CBl04), with apple fiber (ABl04), with banana fiber (BBl04), with passion fruit fiber (PBl04). Yoghurts co-fermented by *B. animalis* subsp. *lactis* B94: control (CB94), with apple fiber (AB94), with banana fiber (BB94), with passion fruit fiber (PB94). Yoghurts co-fermented by *B. animalis* subsp. *lactis* HN019: control (CHN019), with apple fiber (AHN019), with banana fiber (BHN019), with passion fruit fiber (P HN019).

The same trend was observed by Staffolo *et al.* (2004) for yoghurts supplemented with commercial dietary fibers (apple, wheat, bamboo or inulin) without probiotics and by Sendra *et al.* (2008) for citrus fiber yoghurts fermented by different strains of *L. acidophilus*, *Lactobacillus casei* and *Bifidobacterium bifidum* during cold storage. The titration acidity increased at d 14 and kept almost the same at d 28, and the only significant effect in this sense ($P < 0.05$) was that of the passion fruit fiber with the HN019 strain. On the other hand, the yoghurts co-fermented by *L. acidophilus* L10 exhibited the lowest acidity values ($P < 0.05$).

3.3.3. Viable counts

The inoculum levels of starter culture and probiotic strains did not show any significant difference ($P > 0.05$) among the fermentations, being 7.85 ± 0.36 Log CFU mL^{-1} for *Streptococcus thermophilus,* 4.23 ± 0.23 Log CFU mL^{-1} for *Lactobacillus delbrueckii* subsp. *bulgaricus* and 7.75 ± 0.87 Log CFU mL^{-1} for probiotics.

Although Vinderola *et al.* (2002) pointed out the inhibition of *S. thermophilus* growth by some fruit juices, after d 1 the counts of *S. thermophilus* (10.1-11.2 Log CFU mL^{-1}) in apple and banana fibers yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04 and HN019 were significantly higher than in the controls ($P < 0.05$) (Figure 3.2). Oppositely, after d 28 *S. thermophilus* counts decreased to 9.3-10.5 Log CFU mL^{-1} and were higher ($P < 0.05$, $r = 0.652$) in yoghurts co-fermented by *B. animalis* subsp. *lactis* B94 or by *L. acidophilus* L10. Similar results recently reported by Espírito Santo et al. (2010), who also observed higher counts of *S. thermophilus* in yoghurts co-fermented by *L. acidophilus* L10 and *B. animalis* subsp. *lactis* B94, reinforce the positive correlation between *S. thermophilus* and these two probiotic strains. The apparent paradox of a decrease in *S. thermophilus* counts after d 14 and an increase after d 28 is a fact that, indeed, frequently occurs in studies that employ commercial cultures of bacteria, especially those employed in yoghurt making (FLORENCE *et al*., 2009; MARAFON *et al*., 2011), likely because of the presence of a bouquet of bacteria with slight peculiarities in the ability of growth and survive under different conditions of the media. Being so, that group of bacteria grown under the initial conditions and survived likely became old and less adapted to the subsequent changes in pH, acidity and nutrients. Thus, another bacteria group, adapted to the new conditions, grew, survived and became the dominant one.

After d 1, the counts of *L. delbrueckii* subsp. *bulgaricus* (5.0-9.2 Log CFU mL^{-1}) were higher in yoghurts co-fermented by *L. acidophilus* L10 ($P < 0.05$) regardless of the presence of fibers and after the intermediate storage period (d 14); therefore, it is evident that the fibers

supplementation favored its viability. However, after d 28 the highest counts of *L. delbrueckii* subsp. *bulgaricus* were detected in yoghurts co-fermented by *L. acidophilus* L10, regardless of the presence of fibers (Figure 3.3). The same behavior was previously observed in yoghurts inoculated with the same microorganisms, which confirms the positive effect of *L. acidophilus* L10 on *L. delbrueckii* subsp. *bulgaricus* viability (Espírito Santo et al., 2010). Never the addition of fruit fiber at 1% had inhibitory effect on the viability of *L. delbrueckii* subsp. *bulgaricus*, and, in some cases as in yoghurts co-fermented by the *B. animalis* subsp. *lactis* HN019, the presence of apple and banana fibers even stimulated to cell growth compared to control yoghurts after 4 weeks of shelf-life. A symbiotic effect between apple or banana fiber and *B. lactis* HN019 could have been responsible for the enhanced viability of *L. delbrueckii subsp. bulgaricus*. Besides, the low counts of this last microorganism during cold storage was also reported by Marafon *et al*. (2011), who utilized the same starter co-culture (CY340). This observation in two different and independent studies can suggest that the behavior of *L. delbrueckii* subsp. *bulgaricus* is intrinsic to the commercial co-culture characteristics, exhibiting lower counts compared to *S. thermophilus*, maybe as a way to minimize the acetic acid taste produced as the result of *L. delbrueckii* subsp. *bulgaricus* metabolism (SHENE & BRAVO, 2007).

With regard to the probiotic counts, after d 1 both apple and banana fiber yoghurts co-fermented by *L. acidophilus* L10 and the apple fiber one co-fermented by *B. animalis* subsp. *lactis* B94 showed counts (11.5-11.8 Log CFU mL-1) significantly higher than the others ($P < 0.05$), whereas the passion fruit fiber did not exert any significant effect ($P > 0.05$) on probiotic counts (10.6-11.2 Log CFU mL-1) compared to their respective controls (Figure 3.4). After d 14, no significant differences were observed in bifidobacteria counts compared to the controls (Figure 3.4), while a synergistic effect between banana fiber and *L. acidophilus* L10 is evident ($P < 0.05$). Although the longest shelf life (d 28) had a negative impact on the counts of all microorganisms, *L. acidophilus* L10 exhibited by far the lowest values (6.7 to 8.3 Log CFU mL-1), thus suggesting a poor resistance of this strain to the storage conditions. This observation is in accordance with the results of Lamsal and Faubion (2009), who reported lower counts of *L. acidophilus* than of other probiotics in oat enriched products due to its high requirements for several nutrients, whereas Donkor *et al*. (2006) observed a significant decrease of *B. animalis* subsp. *lactis* B94 compared to *L. acidophilus* L10. But the most important observation is that apple and banana fibers increased the counts of all probiotics by no less than 1 Log CFU mL-1 compared to both controls and to passion fruit fiber yoghurts, especially at d 28 (Figure 3.4).

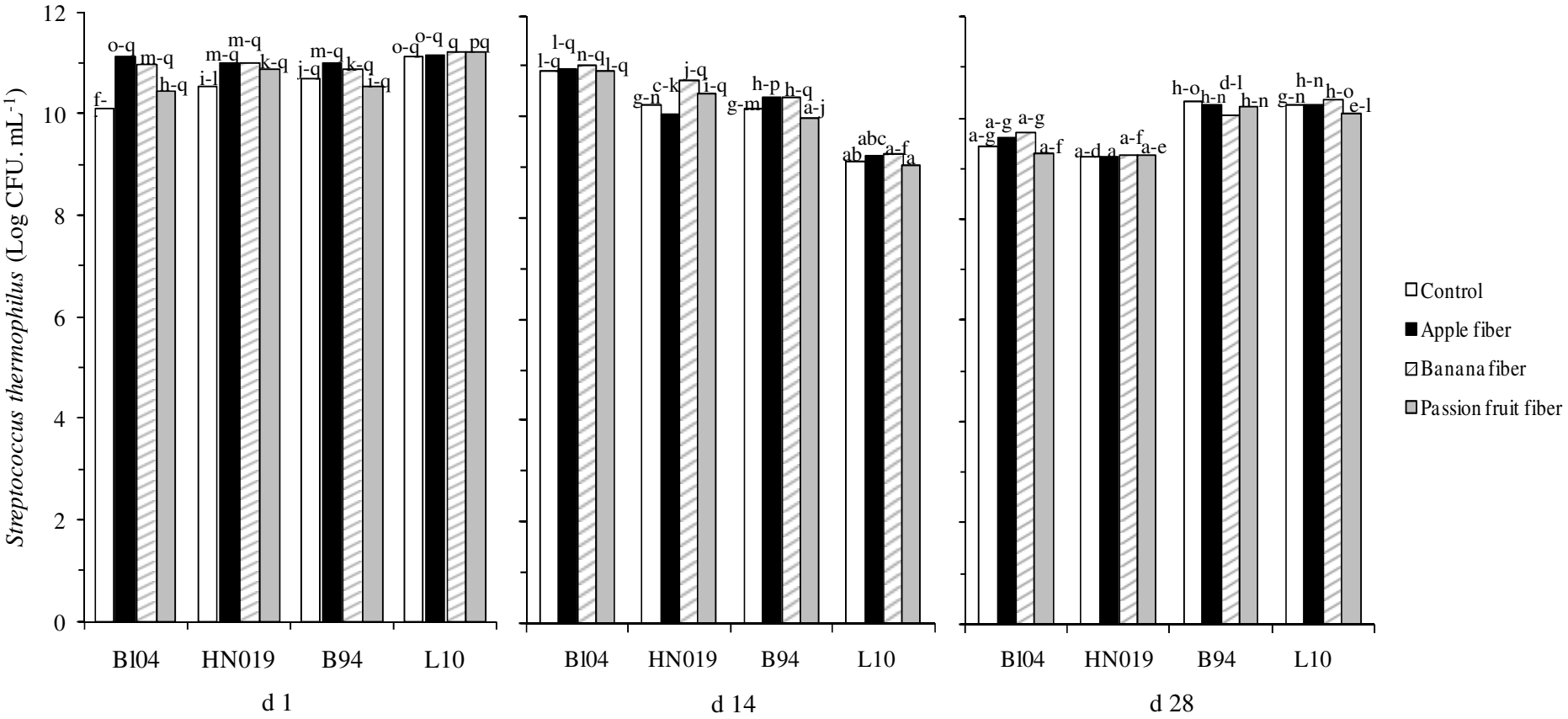

Figure 3.2. *Streptococcus thermophilus* counts in control and fiber yoghurts co-fermented by different probiotic strains.

Abbreviations: Bl04, HN019, B94 = yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04, HN019 and B94, respectively; L10 = yoghurts co-fermented by *L. acidophilus* L10. Means with different letters are significantly different ($P < 0.05$). $N = 40$. d 1, d 14 and d 28 = 1, 14 and 28 days of cold storage.

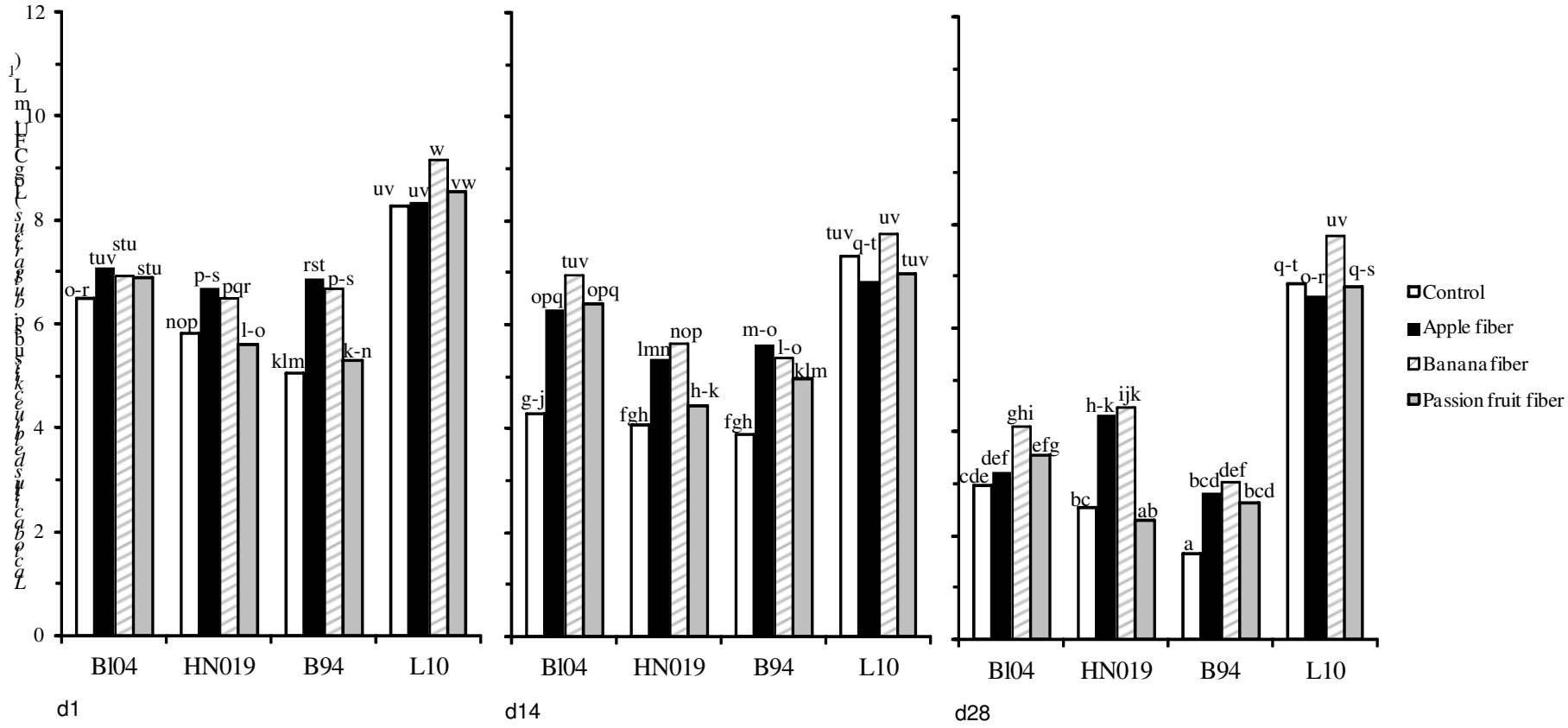

Figure 3.3. *Lactobacillus delbrueckii* subsp. *bulgaricus* counts in control and fiber yoghurts co-fermented by different probiotic strains.

Abbreviations: Bl04, HN019, B94 = yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04, HN019 and B94, respectively; L10 = yoghurts co-fermented by *L. acidophilus* L10. Means with different letters are significantly different ($P < 0.05$). $N = 40$. d 1, d 14 and d 28 = 1, 14 and 28 days of cold storage.

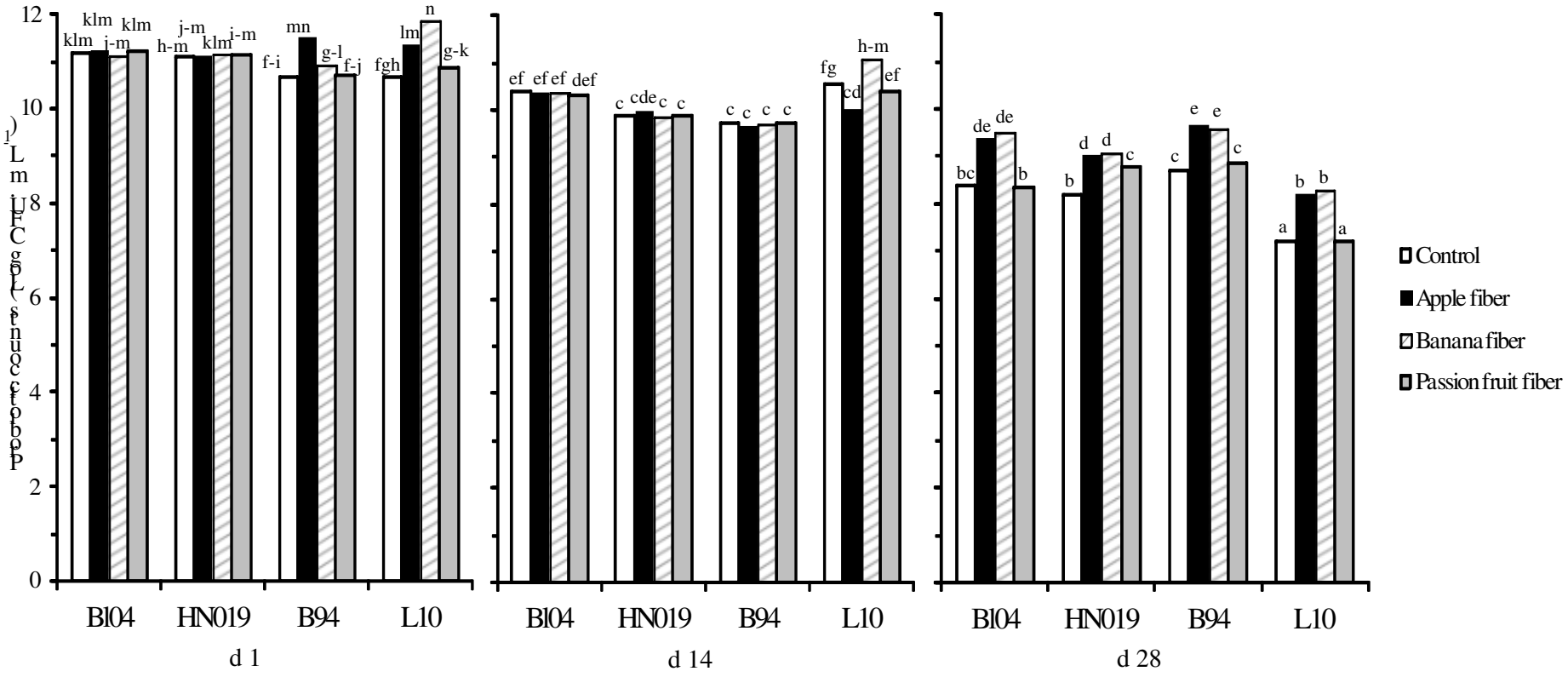

Figure 3.4. Probiotic counts in control and fiber yoghurts co-fermented by different probiotic strains.

Abbreviations: Bl04, HN019, B94 = yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04, HN019 and B94, respectively; L10 = yoghurts co-fermented by *L. acidophilus* L10. Means with different letters are significantly different ($P < 0.05$). $N = 20$. d 1, d 14 and d 28 = 1, 14 and 28 days of cold storage.

These observations point out an *in vitro* symbiotic effect of apple and banana fibers upon the probiotic strains tested, which was similar to those previously observed with orange, inulin, raftilose, maltodextrin, pectin and mainly with fructoolisaccharides on *L. acidophilus* and bifidobacteria (DESAI *et al*., 2004; ÖZER *et al*., 2005; SENDRA *et al*., 2008; OLIVEIRA *et al*., 2009). Based on these observations, one can relate the stimulating effect of both apple and banana fibers on probiotics to their high contents of pectins and fructooligosaccharides (SCHIEBER *et al*., 2001; EMAGA *et al*., 2008). On the other hand, in spite of the presence of pectin (YAPO & KOFFI, 2008), the failure of the passion fruit fiber to increase probiotic counts ($P > 0.05$) could be ascribed to the simultaneous presence of inhibitory compounds such as antibacterial polyphenolics (RIPA *et al*., 2009). In spite of the count variations amongst the probiotic strains, the minimum therapeutic effective dose, which has been suggested to be between 6-9 Log CFU in the product (VASILJEVIC & SHAH, 2008), was reached in all treatments until d 28.

As regards to the possible effect of the different fruit fibers on cell counts, one can see in Figures 3.2 to 3.4 taken as a plain, that the most significant count variations were detected after the longest storage time. In general, whereas no statistically significant effect was observed in all the counts of *S. thermophilus*, those of the other microorganisms decreased according to the following order: banana > apple > passion fruit, being in most cases the effects of the first two fibers statistically coincident. These results can be easily associated to the pectin polymerization degree of these fibers, which was approximately 67, 78, and 93%, respectively (YAPO, 2009; RENARD *et al*., 1995). According to Bazzocco *et al.* (2008), the lower the polymerization degree of a polysaccharide, the higher its prebiotic effect likely because of easier fermentability.

3.3.4. Fatty acids profile

In previous study (ESPIRITO SANTO *et al.,* 2010) the yoghurt starter co-culture CY340, made of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus,* promoted an increase in the content of long chain fatty acids (LCFA) compared to non-fermented milk bases, but it had no effect on those of polyunsaturated fatty acids (PUFA) and CLA even in control yoghurts. Therefore, in the present study, we opted to work with the CY340 always in combination with one probiotic strain.

Table 3.3 shows the fatty acids profiles of skim milk bases, determined after heat treatment. Compared to the other milk bases as well as to the control, the content of short chain fatty acids (SCFA; C4:0 and C6:0) was significantly lower ($P < 0.05$) in banana fiber milk base and that of LCFA (C16:0 to 20:1) complementary higher in milk with apple or banana fiber. On the other hand, fiber addition had lower impact on the content of medium chain fatty acids (MCFA; C8:0 to C15:1).

A rough indication of the fruit fiber fatty acid composition can be obtained by subtracting the data of milk bases with fibers from those without fibers.

According to the saturation degree of the carbon chain, the heat treated milk with apple fiber showed the lowest level of saturated fatty acids (SFA), while the control milk exhibited about one half the PUFA content of all the fiber-containing milks taken as an average ($P < 0.05$). Both effects should be ascribed to the typical compositions of the selected fibers, i.e. to a peculiar abundance of LCFA and PUFA in the banana and apple by-products, respectively (WU *et al*., 2007; EMAGA *et al*., 2008; MOHAPATRA *et al*., 2010). As far as the profiles of the most important fatty acids is concerned, the fiber addition did not lead to any significant variation in the average of stearic (18:0), oleic (18:1) and conjugated linoleic (c9t11-18:2) acids ($P > 0.05$), which were 12.4, 22.5 and 0.71 g/100 g of lipids, respectively (Table 3.3). However, the control milk base exhibited approximately one half the linoleic acid (18:2) content of fiber added milk bases ($P < 0.05$) - which is an important precursor of CLA through the biohydrogenation pathway during lactic acid fermentation (EKINCI *et al.*, 2008) - due to the abundance of such a fatty acid in the fibers (NYANZI *et al*., 2005; WU *et al*., 2007; EMAGA *et al.*, 2008; LIU *et al*., 2008; MOHAPATRA *et al.*, 2010).

The contents of α-linolenic acid (18:3) in the presence of banana, passion fruit and apple fibers were respectively 7, 2 and 1.5-folds those observed in the control milk without fiber ($P < 0.05$), while the eicosanoic acid (20:1) was present in considerable amount only in milk with apple fiber ($P < 0.05$) (Table 3.3). These observations confirm that banana, passion fruit and apple peels are well-known sources of ALA, while the last fiber is also an interesting source of 20:1 (BLANCO-GOMIS *et al*., 2002; ORHAN *et al*., 2002; SONG & BANGERTH, 2003; TOGASHI *et al*., 2007; WU *et al*., 2007; EMAGA *et al*., 2008; MOHAPATRA *et al*., 2010).

The fatty acids profile of the yoghurts remained statistically unchanged after 1, 14 and 28 days of cold storage ($P > 0.05$) (data not shown); so, only the results of d 1 are presented for discussion. This fact may be due to the slower activity of bacteria in yoghurt during cold storage, and the decrease in their viability (Figs. 3.2 to 3.4).

As the starter co-culture (CY340) was present in all samples and exhibited a minor effect on the fatty acids profile in previous work (ESPIRITO SANTO *et al*, 2010), the discussion on this issue was directed only to the effect of the probiotic strains and the fiber type on fatty acids profiles.

Table 3.3. The fatty acids content in g/ 100 g of lipids in total dietetic fibers and heat treated milk bases before fermentation.

Fatty acid	Milk without fiber	Milk with apple fiber	Milk with banana fiber	Milk with passion fruit fiber
SCFA	3.68±0.6^{c}	3.53±0.07^{c}	2.18±0.1^{a}	3.27±0.03^{bc}
4:0	2.50±0.5^{a}	2.49±0.1^{a}	1.06±0.04^{b}	2.24±0.27^{a}
6:0	1.17±0.5^{b}	1.04±0.04^{a}	1.11±0.1^{a}	1.03±0.05^{b}
MCFA	19.8±0.6^{b}	17.9±0.2^{a}	17.3±0.3^{a}	18.7±0.2^{ab}
8:0	1.39±0.4^{a}	1.36±0.1^{a}	0.83±0.04^{b}	1.16±0.1^{a}
10:0	2.33±0.2^{b}	2.02±0.2^{ab}	1.83±0.1^{a}	2.18±0.2^{ab}
12:0	2.80±0.5^{b}	2.49±0.05^{a}	2.48±0.2^{a}	2.71±0.2^{b}
14:0	11.42±0.3^{b}	10.36±0.4^{a}	10.43±0.3^{a}	10.76±0.3^{b}
14:1	0.64±0.01^{a}	0.58±0.02^{a}	0.58±0.01^{a}	0.62±0.02^{a}
15:0	1.22±0.1^{a}	1.08±0.2^{a}	1.12±0.1^{a}	1.24±0.2^{a}
LCFA	76.5±0.6^{a}	78.6±0.5^{bc}	80.6±0.6^{c}	78.0±0.2^{ab}
16:0	34.66±0.8^{a}	32.41±0.2^{b}	36.98±0.7^{c}	33.91±0.6^{a}
16:1	0.57±0.04^{a}	0.49±0.02^{a}	0.61±0.05^{a}	0.56±0.04^{a}
18:0	13.0±0.4^{a}	11.9±0.4^{a}	12.8±0.4^{a}	12.7±0.5^{a}
18:1	22.6±0.7^{a}	22.4±0.8^{a}	22.3±0.8^{a}	22.8±0.6^{a}
18:2	2.42±0.09^{a}	5.82±0.14^{c}	5.31±0.17^{c}	4.15±0.17^{b}
CLA	0.72±0.05^{a}	0.68±0.03^{a}	0.69±0.04^{a}	0.74±0.04^{a}
ALA	0.23±0.03^{a}	0.35±0.03^{b}	1.45±0.08^{d}	0.53±0.05^{c}
20:1	0.00±0.00^{a}	2.60±0.11^{b}	0.00±0.00^{a}	0.00±0.00^{a}
SFA	70.5±1.6^{c}	65.2±1.3^{a}	68.6±0.4^{bc}	68.0±0.3^{b}
MUFA	26.1±0.5^{ab}	28.0±1.2^{ab}	24.9±0.6^{a}	26.3±0.1^{b}
PUFA	3.37±1.16^{a}	6.85±0.08^{c}	6.43±0.22^{bc}	5.70±0.43^{b}

Means ± standard deviation with different letters in the same line are significantly different ($P < 0.05$), $N = 12$.
Abbreviations: short chain fatty acid (SCFA, C4:0 to C6:0); medium chain fatty acid (MCFA, C8:0 to C15:1); long chain fatty acid (LCFA, C16:0 to C18:3). SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid. 18:0, Stearic acid; 18:1, Oleic acid; 18:2, Linoleic acid; 20:1, Eicosanoic acid; ALA, α-Linolenic acid, 18:3; CLA, Conjugated linoleic acid (c9t11-18:2).

Fibers significantly stimulated the production of SCFA ($P < 0.05$) (Table 3.4), which varied, after d 1, in the range 2.75-3.37 g/100 g of lipids in the controls and in the range 3.16-6.08 g/100 g of lipids in fiber yoghurts, respectively. In particular, the banana fiber yoghurt co-fermented by *L. acidophilus* L10 showed the highest fraction of these fatty acids ($P < 0.05$). Edwards and Parret (2002) reported that bacteria from intestinal microbiota ferment unabsorbed carbohydrate to SCFA, which have health benefits related to prevention of heart disease and cancer. A wide range of bowel

bacteria can ferment plant cell-wall biopolymers such as pectins, xylans, arabinogalactans, gums and mucilages into SCFA (MACFARLANE & MACFARLANE, 2003; COSKUN & ONDUL, 2004; YADAV *et al*., 2007). Moreover, *in vitro* experiments demonstrated that bacteria present in the gut are able to ferment individual polysaccharides such starch and pectin into SCFA more rapidly than xylan or arabinogalactan (MACFARLANE & MACFARLANE, 2003). Since the fruit fibers tested in this work are rich in pectins and fructooligosaccharides, they may have provided substrate for the production of SCFA by the probiotic strains.

The contents of MCFA in control yoghurts ranged from 17.34 to 20.41 g/100 g of lipids and were, in general, higher than in those with fruit fiber ($P < 0.05$), in which the MCFA content ranged from 15.67 to 18.47 g/100 g of lipids (Table 3.4).

The LCFA content ranged from 75.22 to 80.51 g/100 g of lipids and constituted the highest lipid fraction in all the yoghurts tested in this work. In general, the fibers promoted the increase in LCFA content of yoghurts compared to the controls, with the exception of banana fiber, as milk containing this supplement showed a LCFA content before fermentation higher than the corresponding yoghurts (Tables 3.3 and 3.4).

These results agree with those found by Espírito Santo et al. (2010), who observed an increase in the production of LCFA in yoghurt co-fermented by the same probiotics in the presence of açai pulp, and pointed out a complex relationship between probiotic strain and fruit ingredient upon the production and transformation of fatty acids.

Figure 3.5 shows that the content of SFA in the control yoghurts ranged from 68.2 to 71.8 g/100 g of lipids and was higher than in fiber yoghurts (61.7 - 68.8 g/100 g of lipids; $P < 0.05$). Such a generalized reduction in the SFA level induced by fibers was more marked in the apple fiber yoghurts, particularly that co-fermented by *L. acidophilus* L10 (12% reduction) ($P < 0.05$).

The highest contents of monounsaturated fatty acids were detected in apple fiber yoghurts regardless of the probiotic strain ($P < 0.05$). Taking into account that the original milk bases showed statistically coincident contents of MUFA ($P > 0.05$) (Table 3.3), this observation suggests the occurrence of a positive interaction between the lipid compounds of the apple fiber and the activity of lactic acid bacteria (Figure 3.5).

Table 3.4. Fatty acids content in g/100 g of lipids in the yoghurts.

Samples	SCFA	MCFA	LCFA	18:0	18:1 (9c)	18:2 (6c)	20:1
CBl04	3.04±0.2^{ab}	17.90±0.2^{de}	78.06±0.2^{cde}	14.00±0.5^{h}	24.45±0.7^{fgh}	2.85±0.1^{b}	0.00±0.0^{a}
CB94	3.37±0.5^{bc}	20.41±0.2^{g}	75.22±0.7^{a}	13.77±0.4^{gh}	21.69±0.5^{a}	2.08±0.1^{a}	0.00±0.0^{a}
CHN019	2.75±0.4^{a}	19.03±0.3^{f}	77.23±0.5^{bc}	12.55±0.3^{cde}	24.80±0.6^{fgh}	3.15±0.1^{b}	0.00±0.0^{a}
CL10	2.76±0.4^{a}	17.34±0.1^{cd}	76.23±0.5^{ab}	13.70±0.4^{gh}	23.19±0.4^{cde}	2.65±0.1^{ab}	0.00±0.0^{a}
ABl04	3.90±0.5^{cde}	16.72±0.2^{abc}	79.37±0.3^{efg}	12.05±0.5^{abc}	23.74±0.8^{def}	6.18±0.3^{fgh}	2.79±0.1^{c}
AB94	4.01±0.3^{de}	17.15±0.4^{cd}	78.83±0.4^{def}	11.91±0.5^{ab}	23.30±0.9^{cde}	6.67±0.3^{hi}	3.15±0.2^{d}
AHN019	3.35±0.6^{bc}	16.81±0.2^{bc}	80.18±0.5^{fg}	11.69±0.3^{a}	24.79±0.8^{h}	6.35±0.3^{ghi}	2.52±0.2^{b}
AL10	4.42±0.5^{e}	15.67±0.3^{a}	79.90±0.6^{fg}	12.61±0.4^{cde}	23.47±0.2^{cef}	6.97±0.3^{i}	3.14±0.3^{d}
BBl04	3.89±0.6^{cde}	17.98±0.2^{def}	80.11±0.7^{fg}	12.66±0.3^{cde}	21.94±0.4^{ab}	5.43±0.1^{e}	0.00±0.0^{a}
BB94	3.38±0.7^{bc}	17.61±0.4^{cd}	80.00±0.7^{fg}	12.89±0.2^{def}	21.82±0.6^{a}	5.53±0.2^{ef}	0.00±0.0^{a}
BHN019	3.16±0.2^{ab}	17.41±0.2^{cd}	80.43±0.4^{g}	12.20±0.5^{abc}	22.41±0.6^{abc}	5.95±0.2^{efg}	0.00±0.0^{a}
BL10	6.08±0.5^{f}	16.84±0.3^{bc}	77.08±0.3^{bc}	12.92±0.4^{def}	21.65±0.2^{a}	6.00±0.2^{efg}	0.00±0.0^{a}
PBl04	3.47±0.5^{bcd}	16.60±0.5^{abc}	79.93±0.4^{fg}	13.98±0.7^{h}	24.53±0.2^{gh}	4.59±0.1^{d}	0.00±0.0^{a}
PB94	4.07±0.2^{e}	18.47±0.2^{ef}	77.26±0.5^{bc}	13.13±0.5^{efg}	22.81±0.2^{bcd}	3.90±0.1^{c}	0.00±0.0^{a}
PHN019	3.96±0.5^{de}	18.03±0.6^{def}	77.97±0.3^{bc}	12.37±0.2^{bcd}	23.09±0.3 ^{bcd}	4.24±0.2^{cd}	0.00±0.0^{a}
PL10	3.91±0.3^{cde}	15.87±0.2^{ab}	80.51±0.4^{g}	13.54±0.3^{fgh}	23.48±0.3^{dfg}	5.47±0.3^{e}	0.00±0.0^{a}

Means ± standard deviation with different letters in the same line are significantly different ($P < 0.05$), $N = 12$.
Abbreviations: short chain fatty acid (SCFA, C4:0 to C6:0); medium chain fatty acid (MCFA, C8:0 to C15:1); long chain fatty acid (LCFA, C16:0 to C18:3). 18:0, Stearic acid; 18:1 (9c), Oleic acid; 18:2 (6c), Linoleic acid; 20:1, Eicosanoic acid. Yoghurts co-fermented by *L. acidophilus* L10: control (CLa), with apple fiber (ALa), with banana fiber (BLa), with passion fruit fiber (PLa). Yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04: control (CBl04), with apple fiber (ABl04), with banana fiber (BBl04), with passion fruit fiber (PBl04). Yoghurts co-fermented by *B. animalis* subsp. *lactis* B94: control (CB94), with apple fiber (AB94), with banana fiber (BB94), with passion fruit fiber (PB94). Yoghurts co-fermented by *B. animalis* subsp. *lactis* HN019: control (CHN019), with apple fiber (AHN019), with banana fiber (BHN019), with passion fruit fiber (P HN019).

Interestingly, all fiber yoghurts showed PUFA contents remarkably higher than their respective controls ($P < 0.05$) (Figure 3.5). Noteworthy is the increase in PUFA content mainly promoted by banana fiber in yoghurts co-fermented by bifidobacteria, in addition to that resulting from the banana fiber addition to the milk base (Table 3.3). On the other hand, the PUFA content was significantly increased by apple and passion fruit fibers in the presence of *L. acidophilus* L10 ($P < 0.05$). Also such significant differences in the PUFA contents could be ascribed to the above-

mentioned synergistic interaction between fruit fiber type and probiotic strain. The increase in unsaturation degree has already been reported for several microorganisms that was suggested to be a universally conserved adaptation response (GUERZONI *et al*., 2001). These results are of great concern because the adhesion of probiotics to the mucosal wall of the distal intestine portion can be promoted by PUFA (KANKAANPÄA *et al*., 2001; DAS & FAMS, 2002). Thus, one should expect that yoghurts containing anyone of the tested fibers could improve the viability of probiotic bacteria and their colonization ability.

In control yoghurts without fibers, the level of CLA (c9 t11-18:2), one of the most important fatty acids for human health, ranged from 0.47 g to 0.80 g/100 g of lipids (Figure 3.6A). The control yoghurts co-fermented by *B. animalis* subsp. *lactis* exhibited CLA content near to the content found in the milk bases, but about 50% higher than those co-fermented by *L. acidophilus* L10, which demonstrates that CLA production is species dependent. Furthermore, the CLA amount in control yoghurt co-fermented by the same strain (0.47 g/100 g of lipids, Figure 3.6A) was lower than in the milk base (0.72 g/100 g of lipids, Table 3.3), showing that in this case part of the initial CLA was transformed in others fatty acids. These observations are supported by the results of previous studies that highlighted different abilities of *Lactobacillus*, *Lactococcus* and *Bifidobacterium* species and strains to produce CLA during milk fermentation (EKINCI *et al*., 2008; OLIVEIRA *et al*., 2009; ESPÍRITO SANTO *et al.,* 2010).

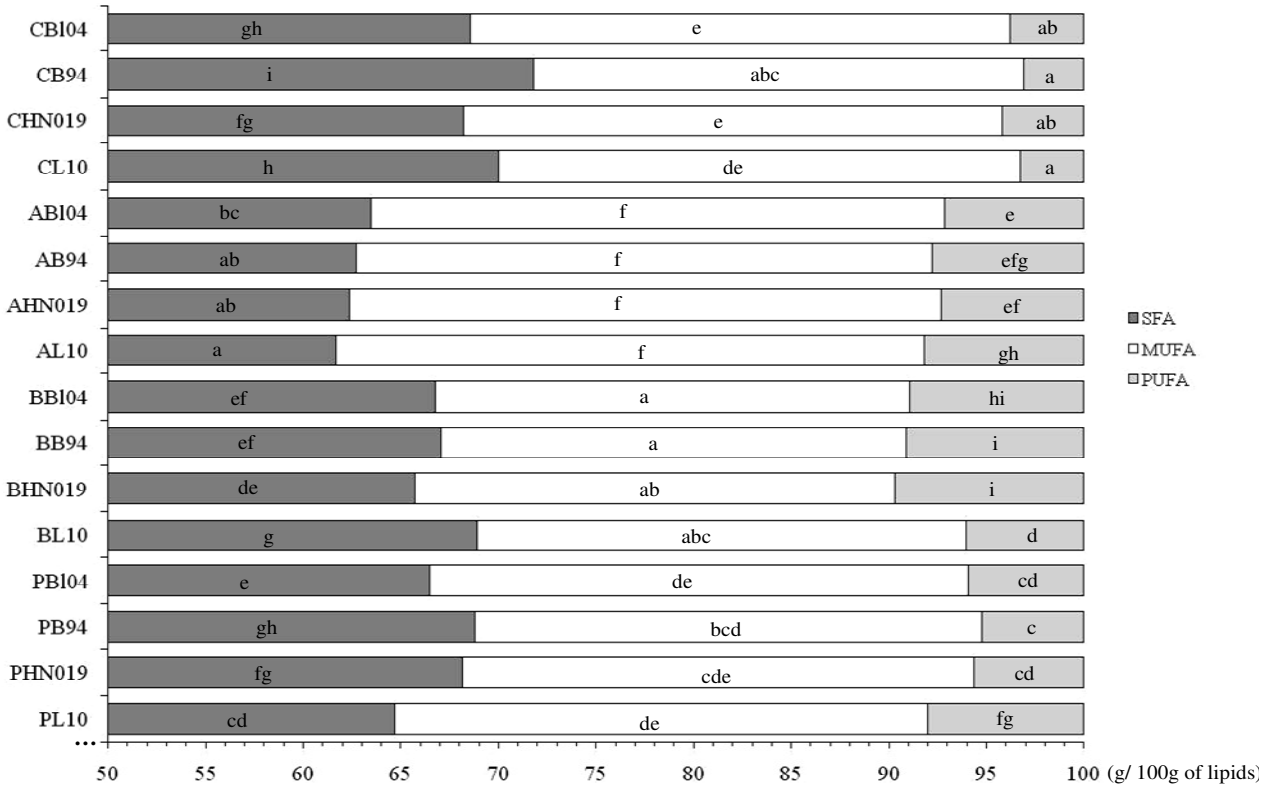

Figure 3.5. Fatty acid profiles (g/100 g of lipids), according to the saturation degree of the carbon chain, observed in control and fiber yoghurts 1 day after the fermentation by different probiotics. Means with different letters are significantly ($P < 0.05$) different. $N = 12$. Abbreviations: SFA = Saturated fatty acid; MUFA = Monounsaturated fatty acid; PUFA = Polyunsaturated fatty acid. Yoghurts co-fermented by *L. acidophilus* L10: control (CL10), with apple fiber (AL10), with banana fiber (BL10), with passion fruit fiber (PL10). Yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04: control (CBl04), with apple fiber (ABl04), with banana fiber (BBl04), with passion fruit fiber (PBl04). Yoghurts co-fermented by *B. animalis* subsp. *lactis* B94: control (CB94), with apple fiber (AB94), with banana fiber (BB94), with passion fruit fiber (PB94). Yoghurts co-fermented by *B. animalis* subsp. *lactis* HN019: control (CHN019), with apple fiber (AHN019), with banana fiber (BHN019), with passion fruit fiber (PHN019).

In fiber yoghurts, the CLA content ranged from 0.54 g to 1.12 g/100 g of lipids and was significantly higher ($P < 0.05$) in those supplemented with the passion fruit fiber (Figure 3.6A). In general, it was observed a negative effect of banana fiber on CLA content in yoghurt co-fermented by *B. animalis* subsp. *lactis* strains compared to the controls. Unexpectedly, this lowering in the CLA amount in banana fiber yoghurts was not followed by the lowering of linoleic acid (18:2), one of the CLA precursors, nor by the increase in the oleic acid (18:0) content, the final product of the biohydrogenation (Table 3.4). In addition, yoghurts containing apple and banana fibers and co-fermented by *B. animalis* subsp. *lactis* B94 exhibited mean CLA values similar to those of their milk bases, suggesting that there was no effect on CLA production during fermentations. Amongst the yoghurts fermented by *B. animalis* subsp. *lactis* HN019, apple and passion fruit fibers led to the highest contents of CLA. But, the most pronounced effect on the CLA content was observed in the presence of *L. acidophilus* L10 (Figure 3.6), which, despite the lowest level of this acid in its control, exhibited the highest increases in fiber yoghurts ($P < 0.05$), especially using the passion fruit and banana fibers, both notoriously rich in pectins. Since the CLA level did not show any statistically significant difference in heat treated milk bases ($P > 0.05$) (Table 3.3) and was the lowest in the control yoghurt co-fermented by *L. acidophilus* L10 ($P < 0.05$) (Figure 3.6), such an effect can certainly be ascribed only to the supplementation and the type of fibers. The generalized higher CLA content in yogurts containing passion fruit fiber compared to controls, in the presence of all the selected strains, is the most evident proof of the possibility of stimulating CLA formation in yoghurts by fruit fiber addition.

However, it is not possible in the present study to suppose a direct relationship between the contents of CLA in fiber yoghurts and of its precursor [linoleic acid (18:2)] in milk bases, because the concentration of linoleic acid was, as an average, significantly higher ($P < 0.05$) in apple and banana fiber milk bases than in passion fruit ones (Table 3.3). As mentioned in the Introduction, the selected dietary fibers are rich sources of many prebiotics, mainly pectins and fructooligosaccharides, which were likely responsible for the observed increases in CLA content. Akalin et al. (2007) and Oliveira et al. (2009) reported that some combinations of probiotic strains, among which *L. acidophilus* and *B. animalis*, and prebiotics such as fructooligosaccharides are able to stimulate the CLA production in fermented milk.

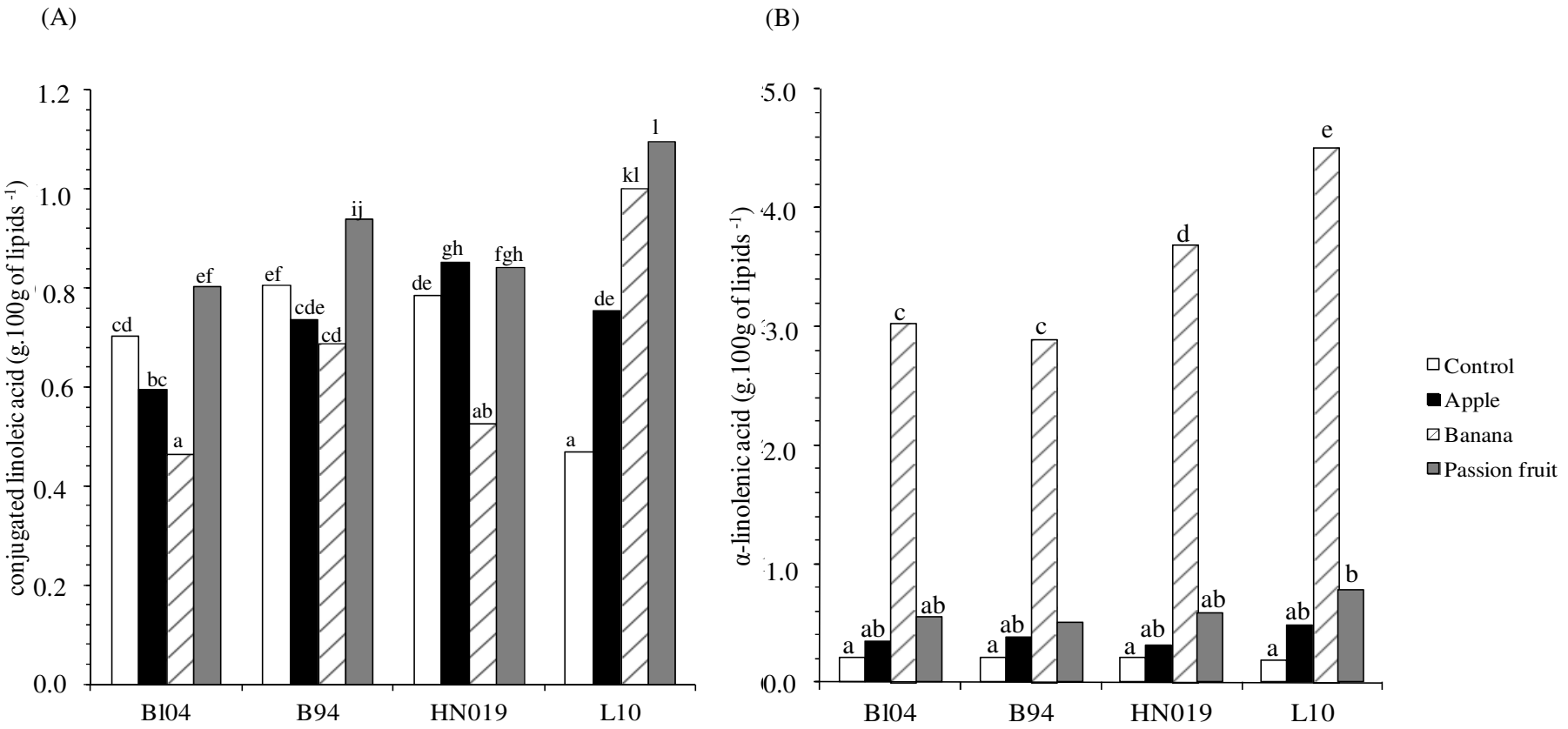

Figure 3.6. Contents of (A) conjugated linoleic acid and (B) α-Linolenic acid in control and fiber yoghurts co-fermented by different probiotic strains. Abbreviations: Bl04, HN019, B94 = yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04, HN019 and B94, respectively; L10 = yoghurts co-fermented by *L. acidophilus* L10. Means with different letters are significantly different ($P < 0.05$). $N = 12$.

These findings on the plain suggest that multiple factors including fiber composition and probiotic strain may be synergistically involved in the CLA production.

Finally, the contents of ALA were around 0.20 g/100 g of lipids in control yoghurts, and ranged from 0.31 g to 4.51 g/100 g of lipids in fiber yoghurts (Figure 3.6B). As a result of the high ALA content of banana peel (EMAGA *et al*., 2008), all the banana fiber yoghurts showed remarkable increases in the level of such polyunsaturated fatty acid with respect to their controls, when compared to the other treatments. An additional synergistic role of the probiotic strains is also suggested by the ALA level that increased, in the presence of this fiber, from 2.8 g/100 g with *B. animalis* subsp. *lactis* Bl04 and B94 to 3.7 g/100 g with *B. animalis subsp. lactis* HN019 and to 4.5 g/100 g of lipids with *L. acidophilus* L10 ($P < 0.05$). The passion fruit fiber also stimulated the ALA production in yoghurts fermented by *L. acidophilus* L10 compared to the control ($P < 0.05$) (Figure 3.6B). These observations are noteworthy since the dietary ingestion of ALA is a factor of inhibition of the proinflammatory cytokine production, which can prevent cardiovascular diseases (ZHAO *et al*., 2007).

3.4. Conclusions

Apple and banana fiber helped to preserve the viability until the fourth week of cold storage of all probiotic strains tested in this study for the production of fiber-enriched skim yoghurts, namely *Bifidobacterium animalis subsp. lactis* Bl04, HN019 and B94 and *Lactobacillus acidophilus* L10. All fiber yoghurts exhibited higher amount of short chain fatty acids and polyunsaturated fatty acids than their controls. There was a synergistic effect between the type of fiber and the probiotic strain on the level of conjugated linoleic acid (c9, t11). Noteworthy, passion fruit fiber promoted CLA increase in all probiotic yoghurts, and all the fruit fibers tested overcame the negative effect of the *L. acidophilus* L10 on the CLA amount in the yoghurts. The content of α-linolenic acid was remarkably increased by the addition of banana fiber. These observations point to the applicability of these processing fruit by-products in the development of new high nutritional value-added probiotic yoghurts.

CHAPTER IV

INFLUENCE OF MILK TYPE AND PASSION FRUIT FIBER ADDITION ON KINETICS, TEXTURE PROFILES AND BACTERIA VIABILITY IN PROBIOTIC YOGHURTS

4.1. Introduction

The passion fruit has origin in tropical countries of America, and Brazil is its greatest producer and consumer, exporting the fruit mainly to United Kingdom, France, Belgium, German and the Netherlands (EMBRAPA, 2010). The cultivation of yellow passion fruit (*Passiflora edulis* var. *flavicarpa* Deg., Passifloraceae) has been preferred for industrial juice production that generates large quantities of by-product composed by seeds and shells representing more than half of the total fruit weight (SALGADO *et al*., 2010).

Functional properties such as anti-hypertensive, hypocholesterolemic and reduction of blood glucose level, have been attributed to the passion fruit peel (CHAU & HUANG, 2005; ZIBADI *et al*., 2007; JANEBRO *et al*., 2008; SALGADO *et al*., 2010). Beyond the content of 10-20% of pectin, a soluble fiber which is known for its prebiotic action, the passion fruit peel is composed of approximately 1.5 g of protein, 0.8 g of lipids, 8.7 g of ash, 56 g of carbohydrates per 100 g of dry matter and is also a source of iron, calcium, phosphorus and niacin (CORDOVA *et al*., 2005; YAPO & KOFFI, 2008). Therefore, it should not be regarded just as an industrial waste, since it can be used for the development of new functional products such as the probiotic ones.

Both dietary fiber and probiotics are reported to relieve constipation and reduce the incidence of colon cancer (KAUR & GUPTA, 2002; FARNWORTH, 2008). In addition, some dietetic fibers from fruit have been recommended as ingredient to probiotic dairy foods because of their beneficial effect on the viability of these bacteria (KOURKOUTAS *et al*, 2006; SENDRA *et al*, 2008; ESPIRITO SANTO *et al*., 2010). However, from the technological point of view the addition of fruit dietetic fiber into a food product with a smooth texture such as yoghurt is a challenge. Both the fermentation and the fragile equilibrium of yoghurt structure can be affected by any fiber added into the milk as well as by the milk type itself (KUMAR & MISHRA, 2003; SODINI *et al.*, 2004, STAFFOLO *et al*., 2004; SENDRA *et al*., 2008). The analysis of the texture profile of yoghurt-like products offers some advantages such as reduced test time and quantification

of structural breakdown, being a useful technique to evaluate the protein gel strength (KUMAR & MISHRA, 2003).

The influence of the milk type and the addition of total dietetic fiber from fruits on kinetics and textural properties of fermented milk products still have been underexploited. This study aimed at evaluating the effect of the milk type and of the total dietetic fiber obtained from passion fruit by-product on the kinetic and texture parameters, post-acidification and microorganism counts of probiotic yoghurts during four weeks of cold storage.

4.2. Materials and Methods

4.2.1. Total dietary fiber preparation

Passion fruit by-product was obtained from an industry of fruit pulp located in the city of Jundiai, São Paulo State, Brazil. The peels of passion fruit were dried in oven under air flow at 60°C until constant weight. The dry peels were reduced to fine powder in a Bimby processor (model TM 31, Vorwerk®, Wuppertal, Germany). In order to make the mixture of the fiber powder into the reconstituted milk easier, the particle size was standardized to less than 42 μm, measured through sieves (Granutest, São Paulo, Brazil). The fiber powder was stored in clapped glass bottles and kept under refrigeration at 4°C until use.

4.2.2. Milk preparation

Skimmed milk Molico® and plain milk Ninho® powders (Nestlé, Araçatuba, Brazil) were both reconstituted to 12% (w/w) in distilled water and each one was divided into two milk samples. Passion fruit fiber was added up to 0.7% to one sample of each of these milks, while the other without fiber was used as control. This percentage of total fiber addition was the highest amount that caused the minimum sineresis by the end of the fermentation (data not shown). All milk bases were heat treated at 85ºC for 15 min under agitation in a water bath and then divided into sterile Schott® flasks (500 mL), cooled in an ice bath, and stored at 4ºC for 24 h.

4.2.3. Microbial cultures

We used in this study a freeze-dried starter yoghurt culture (CY340. DSM, Moorebank, NSW, Australia) - composed of *Streptococcus thermophilus* (St) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb) - and four probiotics, namely two strains of *Lactobacillus acidophilus* (L10. DSM, and NCFM. Danisco, Madison, WI, USA) and two strains of *Bifidobacterium animalis* subsp. *lactis* (Bl04 and HN019. Danisco). The lyophilized cultures were diluted in sterilized milk

and divided into aliquots into Eppendorf® flasks and frozen at -20 °C. Before fermentation, the cultures were thawed and diluted with 50 mL sterilized milk (inoculum). Each Schott® flask containing 500 mL of reconstituted milk was inoculated with 1 mL of yoghurt starter culture with an average count of 8.2 Log CFU mL^{-1} of St and 5.4 Log CFU mL^{-1} of Lb and 1 mL of probiotic culture with counts around 7.4 Log CFU mL^{-1} ($P > 0.05$).

4.2.4. Experimental procedure

Eight different fiber-enriched yoghurts were prepared using the four probiotic strains in the two different milk bases, plus eight controls without fiber. The experimental design is presented in Table 4.1.

After inoculation, the flasks with the samples were transferred to water bath equipment assembled to a CINAC (Cynetique d´acidification, Ysebaert, Frépillon, France) system (Spinnler & Corrieu, 1989), which allows the continuous measurement and recording of pH and the measurement of the four kinetic parameters considered in this study: (a) the maximum acidification rate (V_{max}), expressed in 10^{-3} pH units per min, (b) the time to reach the maximum acidification rate (T_{Vmax}), (c) the time to reach pH 5.0 ($T_{pH5.0}$), near to the isoelectric point of casein and (d) the time to complete the fermentation ($T_{pH\ 4.5}$), all expressed in hours. Two independent batch fermentations were carried out in duplicate on different days at 42ºC up to pH 4.5.

Once the desired pH was reached, the fermentation was interrupted by cooling the flasks to 20°C in an ice bath, and the fermentation time ($T_{pH4.5}$) was recorded. The coagulum was then broken by means of a perforated disk on a stainless steel rod that was moved upwards and downwards for 2 min. The stirred yoghurt was put into 50 mL polypropylene cups, thermally sealed and stored at 4°C.

4.2.5. Total solids, post-acidification and titratable acidity

Determination of total solids in milk bases and titratable acidity in yoghurts were made according to AOAC (1995). The post-acidification was determined as pH after 1, 14 and 28 days of cold storage using a pH meter, model Q-400M1 (Quimis, São Paulo, Brazil). The results were expressed as the means of four replicates.

Table 4.1. Experimental design to evaluate the effect of addition of passion fruit fiber on texture profile and probiotic viability in yogurt made with two types of milk.

Microorganisms (probiotic strain)	Milk type	Passion fruit fiber	[*] Probiotic counts (Log CFU mL^{-1}) in the inocula
[**]Y+ *Lactobacillus acidophilus* (L10)	Skim	-	6.30 ± 0.19
		+	
	Plain	-	
		+	
Y+ *Lactobacillus acidophilus* (NCFM)	Skim	-	6.65 ± 0.22
		+	
	Plain	-	
		+	
Y+ *Bifidobacterium animalis* subsp. *lactis* (Bl04)	Skim	-	6.44 ± 0.31
		+	
	Plain	-	
		+	
Y+ *Bifidobacterium animalis* subsp. *lactis* (HN019)	Skim	-	6.40 ± 0.18
		+	
	Plain	-	
		+	

[*]Means ± standard deviation measured in the inocula, with any statistically difference between them (P >0.05), N = 4.

[**] Co-culture formed by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Y) inocula counts were 8.46 ± 0.32 and 5.02 ± 0.33 Log CFU mL^{-1}, respectively, N = 4.

Abbreviations: without passion fruit fiber (-); with passion fruit fiber (+).

4.2.6. Microbiological analyses

Bacterial enumerations were carried out after 1, 14 and 28 days of cold storage in four replicates of each batch. Samples (1 mL) were diluted with 0.1% sterile peptonated water (9 mL). Afterwards, serial dilutions were carried out, and bacteria were counted, applying the pour plate technique (KODAKA *et al*., 2005). All media were obtained from Oxoid (Basingstoke, UK). In co-cultures, *S. thermophilus* colonies were enumerated in M17 agar, while those of *L. delbrueckii* subsp. *bulgaricus* in MRS (pH 5.4), both under aerobic incubation at 37°C for 48 h. The probiotic microorganisms were incubated at 37°C for 72 h under anaerobic conditions provided by AnaeroGen (Oxoid). Enumerations of *L. acidophilus* were carried out in MRS (pH 6.2) plus 10 μL

mL^{-1} clindamycin, and *Bifidobacterium animalis* subsp. *lactis* in Reinforced Clostridial Agar plus 100 μL mL^{-1} of dicloxacillin. Antibiotics were employed to allow selective growth of the probiotic bacteria. M17 and MRS media (pH 5.4) were prepared according to Jordano *et al.* (1992) and Dave and Shah (1996), and MRS plus clindamycin according to Lankaputhra and Shah (1996). Cell concentration was expressed as Log CFU mL^{-1} of yoghurt.

4.2.7. Texture profile

Texture measurements were carried out as described by Damin *et al. (*2008). Firmness was determined at 4–6ºC by penetration tests made with a TA-XT2 texture analyzer (Stable Micro Systems, Godalming, England) on 50 g packed samples. The probe was a 25 mm diameter acrylic cylinder, moved at a pretest speed of 5 $mm.s^{-1}$, and a test speed of 1 $mm.s^{-1}$ through 10 mm within the sample. The results were expressed as the average of three measurements. Texture properties such as firmness, consistency and cohesiveness were considered. Firmness was measured as the force required to break the structure of yoghurt (RAMCHANDRAN & SHAH, 2009), consistency as the property by which a material (in this case the yoghurt) resists to a change in shape (DEMAN, 1983) and cohesiveness as the extent to which the yoghurt could be deformed before it ruptures (RAWSON & MARSHALL, 1997).

4.2.8. Statistical analyses

The parameters of experimental yogurts were assessed by General Linear Model ANOVA by using Statistica 8.0® software (Statsoft, Tulsa, OK, USA). Different groups were compared by the Tukey test at $P < 0.05$, and statistically significant differences among them were indicated by different letters.

4.3. Results and Discussion

4.3.1. Total solid, pH and kinetics parameters of acidification

The content of total solids of both plain and skim heat treated milk bases without passion fruit fiber was around 13.04 ± 0.12 $g.100\ g^{-1}$, while with fiber was 14.01 ± 0.09 $g.100\ g^{-1}$. As expected, the presence of fiber increased significantly the total solids content of milk bases (by approximately 1%, $P < 0.05$). It was also noticed a significant influence on initial pH that was 6.58

± 0.09 in milk without fiber and 6.42 ± 0.07 in milk with fiber ($P < 0.05$), independently of the milk type.

As Table 4.2 shows, the maximum rate of acidification (V_{max}) was significantly reduced ($P < 0.05$) by the addition of passion fruit fiber in both milk types, which can probably be ascribed to the presence of substances with buffering capacity in the passion fruit fiber, such as organic acids and phenolic compounds (ZIBADI & WATSON, 2004). Furthermore, it was observed that control skim yoghurts co-fermented by *Bifidobacterium* strains exhibited higher V_{max} than the control plain yoghurts co-fermented by the same strains ($P < 0.05$). Nevertheless, the time to reach the maximum acidification rate (T_{max}) was significantly reduced by the presence of the fiber only in plain milk bases and in skims ones co-fermented by lactobacilii. The passion fruit fiber had no effect on the time to reach pH 5.0 ($T_{pH5.0}$) except for the skim yoghurt co-fermented by *L. acidophilus* NCFM, in which the fiber reduced this parameter. Moreover, the time to complete fermentation ($T_{pH5.0}$) in skim control yoghurts co-fermented by *Lactobacillus* strains was longer than in plain ones ($P < 0.05$), thereby indicating a clear effect of the milk type (Table 4.2).

The fermentation lasted from 4.3 to 5.5 h in plain yoghurts and from 5.3 to 6.8 h in skim yoghurts. Considering the milk type, in general the fermentation was quicker in plain milk than in skim milk ($P < 0.05$), while the addition of passion fruit fiber significantly accelerated the fermentation in all skim yoghurts, except that performed by *B. lactis* Bl04. On the other hand, the fiber had no statistically significant effect on $T_{pH4.5}$ in plain yoghurts ($P > 0.05$). The largest reduction of $T_{pH\ 4.5}$ (1 h) due to the passion fruit fiber addition was observed in skim yoghurt fermented by *L. acidophilus* NCFM ($P < 0.05$), although no statistically significant difference ($P > 0.05$), was noticed in the plain yoghurts fermented by the same probiotic strain.

According to Varghese and Mishra (2008), the buffering capacity is directly proportional to the total solids (TS) content of the fermented product, which can lead to longer fermentation time. This observation, which is certainly valid for TS increasing with milk derivatives, does not seem to be applicable to TS increase induced by passion fruit fiber addition that in some cases even accelerated the fermentation (Table 4.2). On the other hand, Almeida *et al.* (2009) ascribed the different acidification profiles of different LABs to their peculiar capacity to assimilate nutritive compounds of the milk, which could explain the differences in the kinetic parameters observed amongst the various yoghurts. In the present study, the correlation analyses indicates that multiple factors, such as the lipid content of the milk, the culture composition and the presence of passion fruit fiber can affect the acidification parameters.

Table 4.2. Kinetic parameters of acidification of plain and skim milks fermented by starter co-culture associated with different probiotic strains with and without passion fruit fiber.

Milk type	Treatment	Probiotic strain	V_{max} (10^{-3}upHmin^{-1})	T_{max} (h)	$T_{pH5.0}$ (h)	$T_{pH\ 4.5}$ (h)
Skim	Control	*L. acidophilus* L10	18.36± 0.01^{d}	2.6± 0.01^{d}	3.35± 0.01^{de}	6.9± 0.29^{h}
		L. acidophilus NCFM	16.69± 0.02^{c}	2.6± 0.01^{d}	3.42± 0.00^{e}	6.6± 0.22^{gh}
		B. lactis Bl04	22.35± 0.03^{e}	2.3± 0.00^{bcd}	2.88± 0.02^{ab}	5.3± 0.19^{cd}
		B. lactis HN019	22.91± 0.02^{e}	2.3± 0.00^{cd}	3.02± 0.01^{a-d}	6.1± 0.24^{fg}
	With passion fruit fiber	*L. acidophilus* L10	13.93± 0.01^{a}	1.9± 0.00^{abc}	3.07± 0.03^{bcd}	6.1± 0.19^{fg}
		L. acidophilus NCFM	14.49± 0.01^{a}	1.9± 0.02^{abc}	3.05± 0.02^{a-d}	5.6± 0.19^{ef}
		B. lactis Bl04	14.02± 0.02^{a}	2.2± 0.01^{bcd}	3.02± 0.02^{a-d}	5.8± 0.17^{fg}
		B. lactis HN019	14.11± 0.02^{a}	2.6± 0.01^{d}	2.98± 0.03^{abc}	5.4± 0.17^{cde}
Plain	Control	*L. acidophilus* L10	17.91± 0.02^{d}	2.3± 0.01^{cd}	3.03± 0.03^{abc}	5.2± 0.16^{bcd}
		L. acidophilus NCFM	16.63± 0.02^{bc}	2.3± 0.00^{bcd}	2.8± 0.04^{ab}	4.4± 0.23^{ab}
		B. lactis Bl04	18.73± 0.01^{d}	2.3± 0.00^{bcd}	2.87± 0.00^{ab}	5.0± 0.20^{abc}
		B. lactis HN019	17.87± 0.01^{d}	2.3± 0.04^{bcd}	2.73± 0.03^{a}	4.3± 0.25^{a}
	With passion fruit fiber	*L. acidophilus* L10	15.66± 0.01^{b}	1.8± 0.01^{a}	3.27± 0.01^{cde}	5.5± 0.29^{def}
		L. acidophilus NCFM	14.10± 0.01^{a}	1.8± 0.00^{a}	3.03± 0.00^{a-d}	4.8± 0.18^{abc}
		B. lactis Bl04	13.90± 0.02^{a}	1.8± 0.03^{a}	2.92± 0.03^{ab}	5.0± 0.27^{abc}
		B. lactis HN019	14.57± 0.03^{a}	1.8± 0.00^{a}	2.77± 0.05^{ab}	4.9± 0.21^{abc}

Means (N = 4) ± standard deviation with different letters in the same column are significantly different (P<0.05). V_{max}, maximum rate of acidification; T_{max}, time to reach V_{max}; $T_{pH5.0}$, time to reach pH 5.0; $T_{pH\ 4.5}$, end time of fermentation.

4.3.2. Pos-acidification and titratable acidity

The results of post-acidification (pH) and titratable acidity during the shelf-life of the yogurts are presented in Table 4.3. After one day of cold storage, the pH of yoghurts ranged from 4.37 to 4.50, and the largest differences between the passion fruit fiber yoghurts and the controls were detected in skim yoghurts fermented by *L. acidophilus* L10 (4.42 fiber yoghurt and 4.50 control) and *B. lactis* Bl04 (4.42 fiber yoghurt and 4.48 control) ($P < 0.05$). Titratable acidity varied from 0.64 to 0.74 mg lactic acid g^{-1} in plain yoghurts and from 0.87 to 1.07 mg lactic acid g^{-1} in skim yoghurts. The increase in this parameter induced by the addition of passion fruit fiber was statistically significant in all yoghurts ($P < 0.05$), but the plain ones co-fermented by *B. lactis* strains.

After 14 days of shelf-life the pH of all yoghurts decreased significantly ($P < 0.05$) and ranged from 4.21 to 4.38 amongst the plain yoghurts and from 4.26 to 4.38 amongst the skim ones. On the other hand, after 28 days, it was observed a slight but significant increase in the average pH of control plain yoghurts co-fermented by *L. acidophilus* NCFM and *B. lactis* strains and passion fruit fiber plain yoghurts co-fermented by *L. acidophilus* strains and *B. lactis* Bl04 ($P < 0.05$). Surprisingly all the passion fruit fiber plain yoghurts showed higher pH than their respective controls ($P < 0.05$). However, such a scenario did in not happen within the skim yoghurts group. In this case the fiber did in fact promote a significant decrease in the pH of all yoghurts, except that co-fermented by *B. lactis* Bl04. A possible explanation of this dual behavior could be the simultaneous occurrence of fatty acid consumption as carbon source after sugar depletion and fiber pectin degradation to uronic acids. Prevalence of the former activity in plain yoghurts was likely responsible for alkalinization, whereas its absence in skim yoghurts led to acidification.

After 14 days of shelf-life all plain yoghurts exhibited a significant increase in their titratable acidity, but they still had lower acidity level compared with the skim yoghurts ($P < 0.05$). At 14 and 28 days the highest values of average titratable acidity were observed in passion fruit fiber skim yoghurts ($P < 0.05$).

Considering the plain period of shelf life, it was observed that the average titratable acidity in yoghurts containing passion fruit fiber was significantly higher than in their respective controls, and that in skim yoghurts higher than in the plain ones ($P < 0.05$). As far as the probiotic cultures is concerned, in general, the yoghurts co-fermented by *L. acidophilus* strains exhibited lower titratable acidity than those co-fermented by *B. lactis* strains ($P < 0.05$). Such a behavior should be indeed expected by the fact that the homolactic metabolism of the former leads to two lactic acid moles per

mole of glucose consumed, while that of bifidobacteria to one mol of lactic acid and 1.5 moles of acetic acid.

Table 4.3. Post-acidification (pH) and titratable acidity during shelf-life of control and passion fruit fiber yogurts.

Yoghurts			pH *			Titratable acidity (% lactic acid) *		
Milk type	**Treatment**	**Probiotic strain**	**d 1**	**d 14**	**d 28**	**d 1**	**d 14**	**d 28**
Skim	Control	La L10	4.50 ^{x}	4.38 ^{opqr}	4.40 ^{pqrs}	0.87 ^{ij}	0.95 ^{k}	0.96 ^{k}
		La NCFM	4.43^{stu}	4.34 ^{klm}	4.40 ^{pqrs}	0.90 ^{j}	0.98 ^{kl}	1.02 ^{lmn}
		Bl04	4.48 ^{vx}	4.32 ^{hijk}	4.34 ^{lmn}	0.96 ^{k}	0.98 ^{kl}	1.18 st
		Bl HN019	4.43 ^{tu}	4.28 ^{efg}	4.39 ^{pqr}	0.95 ^{k}	0.99 ^{klm}	1.14 ^{rs}
	With passion	La L10	4.43 ^{stu}	4.26^{bcd}	4.33 ^{hijk}	0.97 ^{k}	1.09 ^{pq}	1.10 ^{pq}
	fruit fiber	La NCFM	4.45 ^{tuv}	4.30 ^{ghi}	4.34 ^{jklm}	0.97 ^{k}	1.04 ^{no}	1.08 ^{op}
		Bl04	4.42 ^{stu}	4.29 ^{efg}	4.34 ^{jklm}	1.07 ^{nop}	1.10 ^{pq}	1.22 ^{tu}
		Bl HN019	4.47 ^{uvx}	4.30 ^{fgh}	4.32 ^{hij}	1.04 ^{mn}	1.08 ^{op}	1.25 ^{u}
Plain	Control	La L10	4.39 ^{opqr}	4.23 ^{ab}	4.23 ^{abc}	0.66 ^{a}	0.77 ^{efg}	0.74 ^{de}
		La NCFM	4.42 ^{rst}	4.21 ^{a}	4.22 ^{ab}	0.65 ^{a}	0.78 ^{gh}	0.76 ^{ef}
		Bl04	4.37 ^{lmn}	4.24 ^{abc}	4.22 ^{ab}	0.67 ^{ab}	0.76 ^{ef}	0.76 ^{ed}
		Bl HN019	4.44 ^{tuv}	4.28 ^{efg}	4.26 ^{cde}	0.64 ^{a}	0.72 ^{cd}	0.73 ^{cd}
	With passion	La L10	4.37 ^{lmno}	4.29 ^{fgh}	4.32 ^{fghi}	0.68 ^{a}	0.74 ^{de}	0.74 ^{de}
	fruit fiber	La NCFM	4.46 ^{uv}	4.35 ^{lmno}	4.38 ^{nop}	0.65 ^{ab}	0.74 ^{de}	0.70 ^{bc}
		Bl04	4.41 ^{rst}	4.32 ^{fghi}	4.31 ^{ghij}	0.75 ^{def}	0.80 ^{hi}	0.78 ^{ghi}
		Bl HN019	4.42 ^{stu}	4.34 ^{klm}	4.33 ^{ijkl}	0.72 ^{cd}	0.80 ^{hi}	0.77 ^{efg}

* Standard deviations were under 0.05. Means (N = 4) with different letters in the same column are significantly different (P<0.05). Abbreviations: d1, d14 and d28 = days 1, 14 and 28 after fermentation. La NCFM and LaL10, *L. acidophilus* strains NCFM and L10; Bl HN019 and Bl04, *B. animalis* subsp. *lactis* strains HN019 and Bl04.

4.3.3. Microorganisms viability

During the plain shelf-life, *S. thermophilus* counts were stable and ranged, as an average, from 8.6 to 10.9 Log CFU mL^{-1} (Figure 4.1). In the period between 1 and 14 days, a mild but significant decrease in St counts occurred in all yoghurts co-fermented by *L. acidophilus* strains, but an increase in skim yoghurts co-fermented by *B. lactis* strains ($P < 0.05$).

In contrast with St counts invariability during shelf-life, *L. delbrueckii* subsp. *bulgaricus* suffered a large decrease in its counts, which ranged from 6.2 to 9.5 and from 2.9 to 7.1 Log CFU mL^{-1} after 1 and 28 days, respectively (Figure 4.2). At the end of the plain shelf-life, the highest counts of Lb were observed in yoghurts co-fermented by *L. acidophilus* strains, particularly the L10 one ($P < 0.05$). Such a synbiotic effect of *L. acidophilus* L10 on Lb was previously noticed by Espirito Santo *et al* (2010).

At the 1st day of cold storage, the probiotic counts varied from 8.5 to 10.8 Log CFU mL^{-1} in yoghurts co-fermented by *L. acidophilus* strains and from 7.9 to 9.9 Log CFU mL^{-1} by *B. lactis* strains (Figure 4.3). Amongst the skim yoghurts, the counts of *L. acidophilus* were about 1 Log higher than those of *B. lactis* ($P < 0.05$) in spite of the same counts of both probiotic species in the inocula. Regarding the control, a beneficial effect of passion fruit fiber was observed only in *B. lactis* Bl04 counts in skim yoghurt, but the contrary took place in plain yoghurt ($P < 0.05$).

A dramatic change in the probiotic counts profile in skim yoghurts occurred after 14 days of shelf-life. The counts of *B. lactis* raised by 1.5 Log as an average and were significantly higher than the ones of *L. acidophilus* that decreased by about 2 Log ($P < 0.05$). Furthermore, the passion fruit fiber had a beneficial effect on the counts of *B. lactis* strains in skim yoghurts and those of *B. lactis* HN019 in plain yoghurt ($P < 0.05$), the only negative effect of the fiber being detected in the counts of *L. acidophilus* NCFM in plain yoghurts ($P < 0.05$) (Figure 4.3).

At the end of shelf-life, the counts of the probiotic strains ranged, as a plain, from 6.4 to 8.9 Log CFU mL^{-1}, being higher in skim yoghurts except for *L. acidophilus* L10 on which no effect due to milk type was observed. The passion fruit fiber did not promote any significant variation in the probiotic counts, except in that of *B. lactis* Bl04 in plain yoghurt that was 0.8 Log higher than its control.

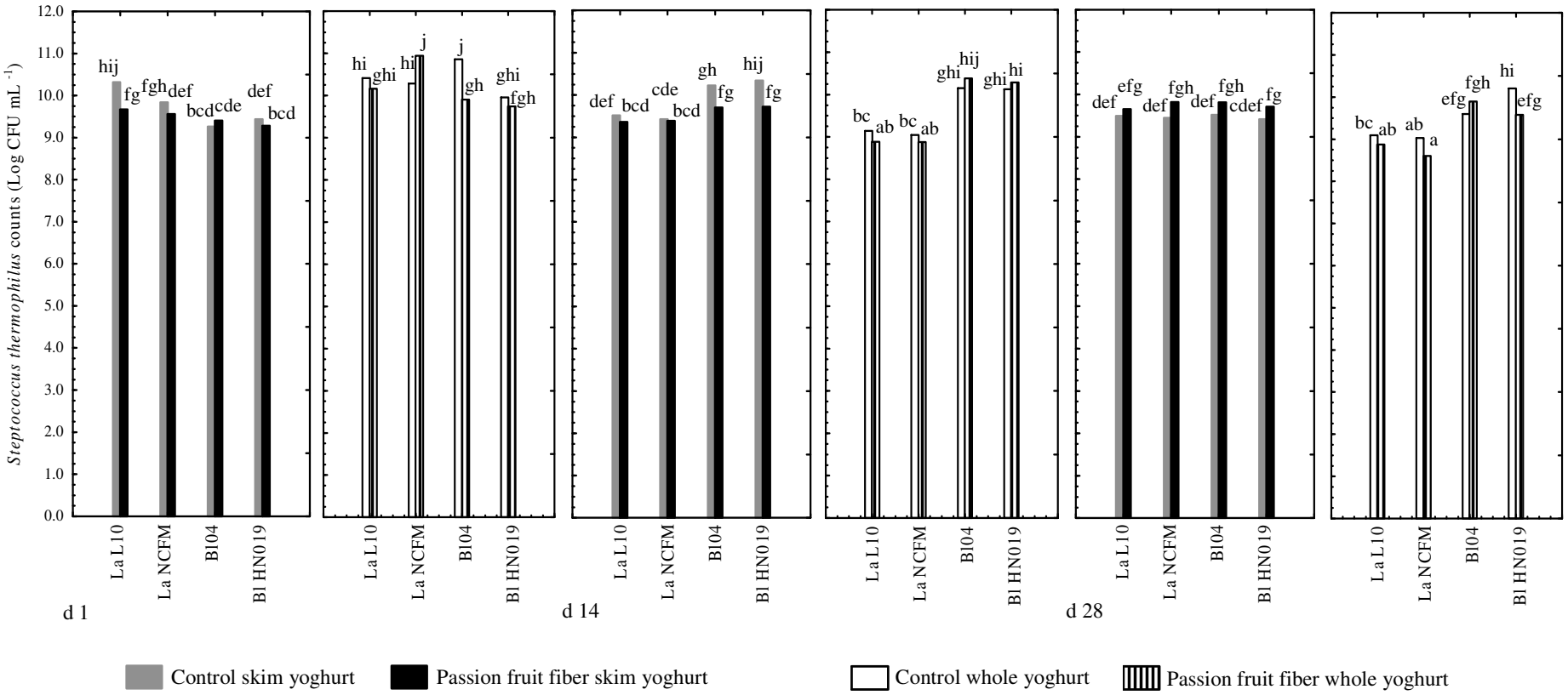

Figure 4.1. *Streptococcus thermophilus* counts in control and fiber yoghurts co-fermented by different probiotic strains.

Abbreviations: Bl04, HN019, B94 = yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04, HN019 and B94, respectively; L10 = yoghurts co-fermented by *L. acidophilus* L10. d1, d14 and d28 = days 1, 14 and 28 after fermentation. Means with different letters are significantly different ($P < 0.05$). $N = 64$.

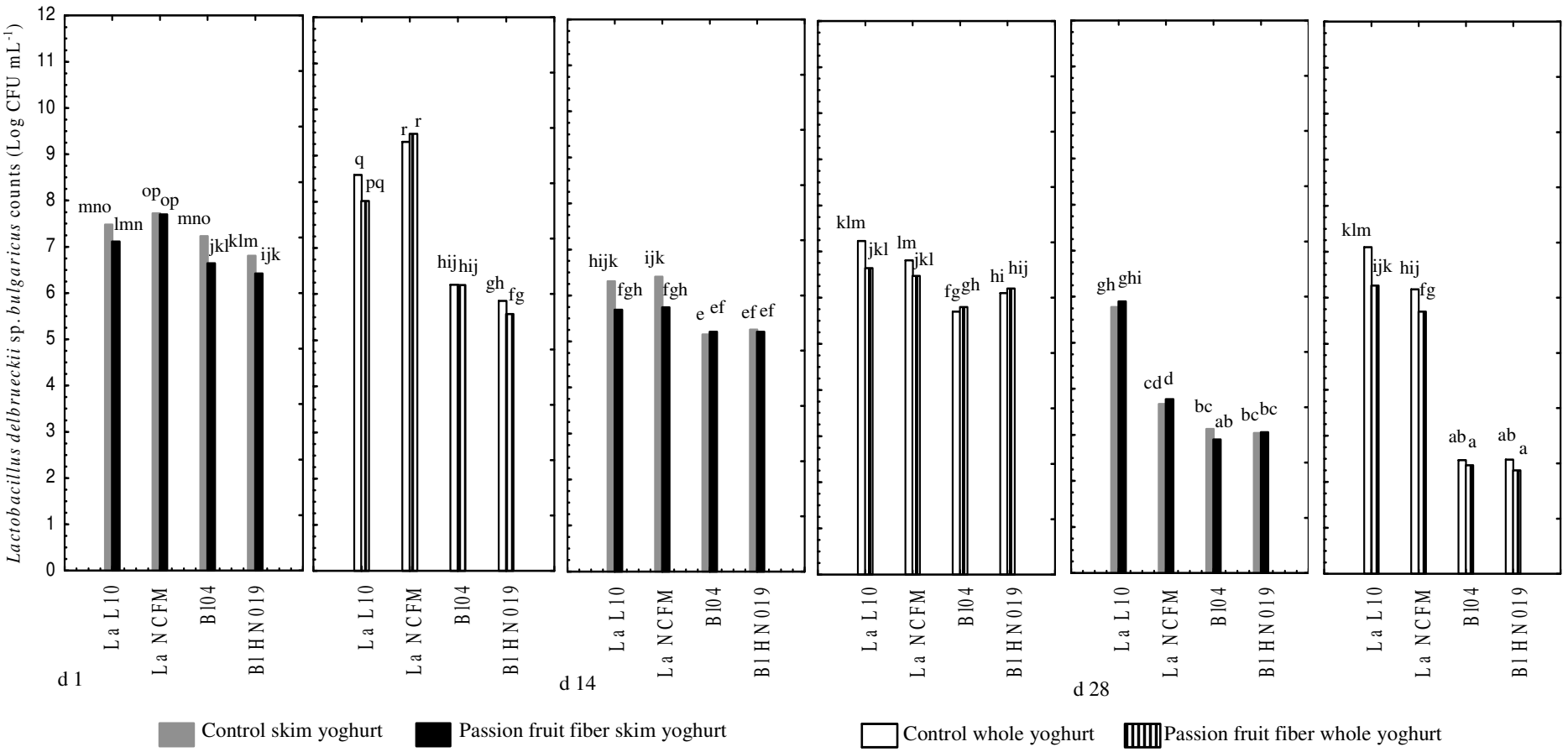

Figure 4.2. *Lactobacillus delbrueckii* subsp. *bulgaricus* counts in control and fiber yoghurts co-fermented by different probiotic strains.

Abbreviations: Bl04, HN019, B94 = yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04, HN019 and B94, respectively; L10 = yoghurts co-fermented by *L. acidophilus* L10. d1, d14 and d28 = days 1, 14 and 28 after fermentation. Means with different letters are significantly different ($P < 0.05$). $N = 64$.

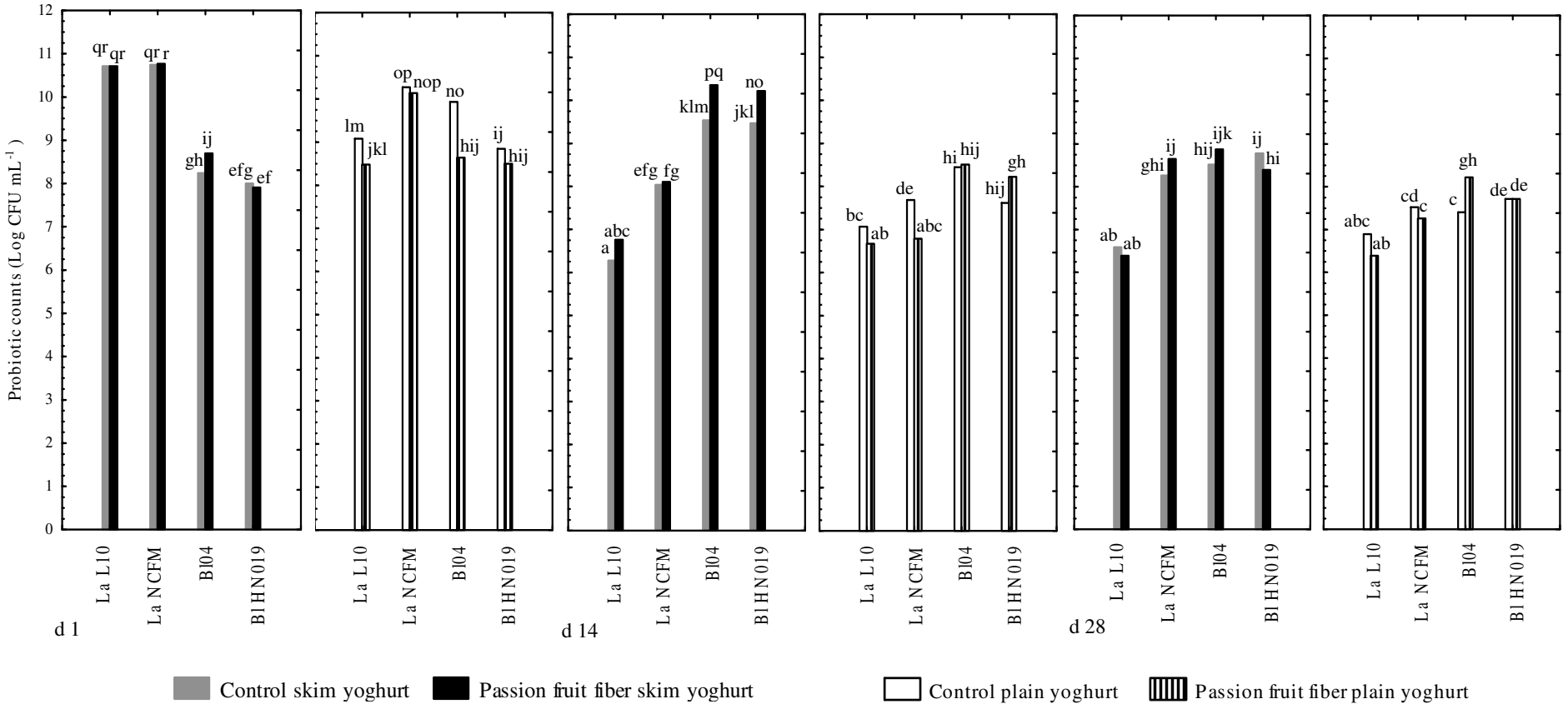

Figure 4.3. Probiotic counts in control and fiber yoghurts co-fermented by different probiotic strains.

Abbreviations: Bl04, HN019, B94 = yoghurts co-fermented by *B. animalis* subsp. *lactis* Bl04, HN019 and B94, respectively; L10 = yoghurts co-fermented by *L. acidophilus* L10. d1, d14 and d28 = days 1, 14 and 28 after fermentation. Means with different letters are significantly different ($P < 0.05$). $N = 16$.

Talcott *et al*. (2003) and Narain *et al.* (2004) reported that some compounds of passion fruit, such as phenolic compounds, fatty acid esters, thiols, terpenes and alcohols can inhibit the growth of *L. acidophilus*. According to a study of Vinderola *et al.* (2002), the strawberry, pineapple and kiwi juices did not influence the growth of *L. acidophilus* when the juices were previously neutralized. Likewise, the initial pH of the milk containing passion fruit fiber – which was near the neutrality (pH 6.42) - may have attenuated the possible negative effect of the acidity from the fruit on the viability of *L. acidophilus* and *B. lactis* strains tested. Besides, the concentration of passion fruit fiber may not have been enough to exert an inhibitory effect on the probiotics, with exception of the NCFM strain on the 14th day.

4.3.4. Texture profile

The texture profiles of the different yoghurts evaluated after 1, 14 and 28 days of cold storage are shown in Table 4.4.

Regarding only the influence of the milk type, during the cold storage the plain control yoghurts co-fermented by lactobacilii showed higher firmness, consistency and cohesiveness than the respective skim ones ($P < 0.05$). This observation is supported by some studies that pointed out that a reduction in fat content can cause a fragile texture due to weaker network of the protein gel in yoghurts (GUVEN *et al.,* 2005; RAMCHANDRAN & SHAH, 2009). As far as the influence of passion fruit fiber is concerned, it promoted, as an average, higher values of all texture parameters in skim yoghurts co-fermented by *B. lactis* strains compared to their respective controls or to plain fiber yoghurts ($P < 0.05$). Meanwhile, it reduced significantly the firmness and consistency, but not the cohesiveness, of plain yoghurts co-fermented by *L. acidophilus* L10.

As expected, in general, all texture parameters significantly increased during cold storage, being the most marked increase observed after 1 and 14 days. At the end of storage, firmness and consistency in all passion fruit fiber skim yoghurts were higher than in their respective controls, except when using *L. acidophilus* NCFM as probiotic, while their cohesiveness was increased by the addition of fiber in all cases. As regards the plain yoghurts, firmness was higher in controls co-fermented by *L. acidophilus* NCFM and *B. lactis* strains ($P < 0.05$), while consistency and cohesiveness were significantly higher in the same yoghurts but that co-fermented by *B. lactis* Bl04.

Table 4.4. Texture parameters of plain and skim yogurts co-fermented by different probiotic strains and with or without passion fruit fiber

Yoghurts			Firmness (N)			Consistency (N.s)			Cohesiveness (N)		
Milk type	**Treatment**	**Probiotic**	**d 1**	**d 14**	**d 28**	**d 1**	**d 14**	**d 28**	**d 1**	**d 14**	**d 28**
Skim	Control	La L10	0.25 ^{a}	0.28 ^{ab}	0.37 ^{cd}	2.06 ^{ab}	2.19 ^{bc}	2.60 ^{cd}	0.16 ^{a}	0.22 ^{ab}	0.23 ^{ab}
		La NCFM	0.25 ^{a}	0.31 ^{bc}	0.35 ^{cd}	1.95 ^{a}	2.56 ^{c}	2.69 ^{cd}	0.19 ^{a}	0.25 ^{abc}	0.24 ^{c}
		Bl04	0.27 ^{ab}	0.36 ^{cd}	0.39 ^{cde}	2.22 ^{abc}	2.70 ^{d}	2.90 ^{de}	0.22 ^{ab}	0.27 ^{bc}	0.26 ^{bc}
		Bl HN019	0.27 ^{bc}	0.41 ^{de}	0.37 ^{cd}	2.56 ^{c}	3.08 ^{de}	2.82 ^{cde}	0.25 ^{abcd}	0.30 ^{cd}	0.26 ^{bc}
	With passion	La L10	0.37 ^{d}	0.45 ^{efg}	0.48 ^{fg}	2.95 ^{de}	3.50 ^{f}	3.51 ^{f}	0.31 ^{cde}	0.36 ^{def}	0.38 ^{efg}
	fruit fiber	La NCFM	0.27 ^{ab}	0.38 ^{cd}	0.38 ^{cde}	2.15 ^{ab}	2.94 ^{e}	2.94 ^{e}	0.22 ^{ab}	0.31 ^{cde}	0.30 ^{cd}
		Bl04	0.43 ^{e}	0.50 ^{g}	0.48 ^{fg}	3.39 ^{ef}	3.82 ^{g}	3.58 ^{f}	0.36 ^{e}	0.40 ^{g}	0.37 ^{ef}
		Bl HN019	0.37 ^{d}	0.48 ^{fg}	0.48 ^{fg}	3.49 ^{f}	3.97 ^{gh}	3.50 ^{f}	0.32 ^{de}	0.38 ^{efg}	0.36 ^{def}
Plain	Control	La L10	0.34 ^{cd}	0.45 ^{ef}	0.44 ^{ef}	2.86 ^{cde}	3.55 ^{f}	3.53 ^{f}	0.28 ^{bc}	0.31 ^{cde}	0.32 ^{de}
		La NCFM	0.31 ^{bc}	0.49 ^{fg}	0.52 ^{g}	2.63 ^{cd}	4.06 ^{gh}	4.18 ^{h}	0.26 ^{bc}	0.35 ^{def}	0.37 ^{ef}
		Bl04	0.28 ^{abc}	0.31 ^{bc}	0.49 ^{fg}	2.23 ^{bc}	2.39 ^{bcd}	3.05 ^{de}	0.23 ^{ab}	0.21 ^{ab}	0.29 ^{cd}
		Bl HN019	0.26 ^{ab}	0.32 ^{bc}	0.51 ^{g}	2.11 ^{ab}	2.35 ^{bcd}	3.28 ^{ef}	0.21 ^{ab}	0.20 ^{a}	0.36 ^{def}
	With passion	La L10	0.24 ^{a}	0.39 ^{de}	0.51 ^{g}	1.59 ^{a}	2.58 ^{cd}	3.28 ^{ef}	0.22 ^{ab}	0.24 ^{abc}	0.36 ^{def}
	fruit fiber	La NCFM	0.28 ^{abc}	0.40 ^{de}	0.47 ^{efg}	1.50 ^{a}	2.86 ^{cde}	3.05 ^{de}	0.26 ^{bc}	0.27 ^{bc}	0.29 ^{cd}
		Bl04	0.28 ^{ab}	0.38 ^{de}	0.37 ^{cd}	2.11 ^{ab}	2.82 ^{cde}	2.72 ^{d}	0.23 ^{ab}	0.28 ^{bc}	0.29 ^{bcd}
		Bl HN019	0.28 ^{abc}	0.38 ^{de}	0.38 ^{cde}	2.15 ^{ab}	2.61 ^{cd}	2.79 ^{de}	0.23 ^{ab}	0.26 ^{bc}	0.27 ^{bc}

Means with different letters in the same column are significantly different ($P < 0.05$). $N = 12$. All standard deviations were under 5% of the average and are not shown.
Abbreviations: D1, D14 and D28 = days 1, 14 and 28 after fermentation. LaL10 and La NCFM, *L. acidophilus* strains L10 and NCFM; Bl04 and Bl HN019, *B. animalis* subsp. *lactis* strains Bl04 and HN019.

According to Damin *et al.* (2008), the firmness is higher in yoghurts lasting longer fermentation time. However, in the present study skim yoghurts co-fermented by lactobacilli - in spite of the longer fermentation time - did not show any firmness increase after 1 day of cold storage compared to the other treatments.

Cultures of lactic acid bacteria producer of exopolysaccharides (EPS) have been used to improve the texture of yoghurts (WELMAN & MADDOX, 2003; SODINI *et al.*, 2004). However, the high counts of EPS-producing *L. acidophilus* and *S. thermophilus* in skim yoghurts did not correspond to any increase in their textural parameters. This observation can be explained with the formation of a few weak polysaccharide–protein interactions instead of more stable protein-protein ones (FOLKENBERG *et al.*, 2006; RAMCHANDRAN & SHAH, 2009), which may have contributed to lowering the firmness of yoghurts.

The results of the present study taken together suggest that the textural parameters were influenced by a combination of factors such as culture composition, milk type and passion fruit fiber addition, which justifies further efforts in this field.

4.4. Conclusions

The effects of milk type and passion fruit fiber addition on the kinetic and texture parameters, post-acidification and microorganisms counts were investigated in yoghurts co-fermented by a common starter culture and one of the following probiotics, *Lactobacillus acidophilus* (strains L10 and NCFM) and *Bifidobacterium animalis* subsp. *lactis* (strains Bl04 and HN019). The most remarkable findings of this study are:

- the passion fruit fiber reduced significantly the maximum acidification rate in both skim and plain milks and accelerated the fermentation in all skim yoghurts, except the one co-fermented by *B. lactis* Bl04;
- the titratable acidity was higher in passion fruit fiber yoghurts and in skim control yoghurts;
- *L. acidophilus* strains exerted a synbiotic effect on *L. delbrueckii* subsp. *bulgaricus*;
- contrary to the milk type, the passion fruit fiber did not show any clear effect on probiotic counts during the shelf-life;
- the addition of passion fruit fiber increased the cohesiveness of all probiotic skim yoghurts at the end of shelf-life.

CONCLUSÕES

Este estudo traz evidências inéditas, entre elas as principais são:

O efeito prebiótico da polpa de açaí sobre a viabilidade de *L. acidophilus* L10, *Bifidobacterium animalis subsp. lactis* Bl04 e *B. longum* Bl05 em iogurtes na quarta semana de armazenamento refrigerado;

A polpa de açaí combinada a *Lactobacillus acidophilus* L10, *B. lactis Bifidobacterium* Bl04 ou B94, age sinergisticamente sobre a viabilidade de *Streptococcus thermophilus* nos iogurtes;

As fibras de maçã e banana exerceram um efeito prebiótico em todas as cepas testadas: *L. acidophilus* L10 e *B. lactis* Bl04, HN019 e B94;

Tanto a polpa de açaí quanto as fibras dietéticas totais de maçã, banana ou maracujá estimularam a produção de ácidos graxos poliinsaturados e os de cadeia curta e reduziram o teor dos saturados em relação aos respectivos controles, tornando mais saudável a fração lipídica dos iogurtes;

A combinação de adição de polpa de açaí e algumas cepas probióticas, particularmente de *B. lactis* cepas Bl04 e B94, aumenta a produção de ácido linoleico conjugado (CLA) nos iogurtes;

As fibras de maracujá estimularam a produção de CLA e promoveram a coesividade de todos os iogurtes desnatados.

Em suma, os resultados apontam a aplicabilidade tanto do uso da polpa de açaí quanto das fibras dietéticas totais dos subprodutos da maçã, banana e maracujá como ingredientes capazes de melhorar o perfil lipídico de iogurtes desnatados, o que leva à formulação de um produto alimentar com elevado valor agregado, seja do ponto de vista da saúde do consumidor, quanto do ponto de vista ambiental.

Através desta tese surgiram questões que constituem oportunidades para estudos futuros, tais como:

Elucidar os compostos presentes no açaí e na fibra de maracujá capazes de estimular a produção de CLA e outros ácidos graxos poliinsaturados pelos micoorganismos testados, especialmente por *B. lactis* cepas Bl04 e B94;

Determinar os fatores presentes na polpa de açaí responsáveis por estimular especificamente a viabilidade de *L. acidophilus* L10, *B. lactis* Bl04 e *B. longum* Bl05 na quarta semana de armazenamento a frio;

De modo semelhante, avaliar os fatores presentes nas fibras de maçã e de banana capazes de estimular a viabilidade de *L. acidophilus* L10, *B. lactis* Bl04, B94 e HN019.

CONCLUSIONS

The main conclusions of this study are:

The açaí pulp presented a prebiotic effect on the viability of *L. acidophilus* L10, *B.* lactis Bl04 and *B. longum* Bl05 in yoghurt at 4 weeks of storage;

The acai pulp in combination with *Lactobacillus acidophilus* L10, *B. lactis* Bl04 or B94 act synergistically on the viability of *Streptococcus thermophilus*;

The prebiotic effect was also observed in apple and banana fibers on the viability of *L. acidophilus* L10 and strains of *B. lactis* Bl04, HN019 and B94;

Both açaí pulp and total dietary fiber of apple, banana and passion fruit added into milk, stimulated the production of polyunsaturated and short chain fatty acids and reduced the content of saturated ones in yoghurts in respect to their controls, making healthier the lipid fraction of the yogurt;

Besides, the combination of acai pulp and some probiotic strains, particularly *B. lactis* Bl04 and B94 increases the production of conjugated linoleic acid (CLA) in yogurt;

The passion fruit fibers stimulated the production of CLA and promoted the cohesiveness in all skim yoghurt.

In sum, the results show the applicability of both the acai pulp and the total dietary fiber from by-products of apple, banana and passion fruit as ingredients that can improve the lipid profile of yoghurt, which can lead to formulations of high value added food products, from both the consumer health and the environmental points of view.

Through this thesis some questions that arisen constitute opportunities for future studies such as:

Elucidate the compounds present in the açaí and passion fruit fiber that are able to stimulate the production of CLA and other polyunsaturated fatty acids by the micoorganisms tested, especially by *B. lactis* strains Bl04 and B94;

Determine the factors present in the açaí yoghurts specifically responsible for stimulating the viability of *L. acidophilus* L10, *B. lactis* Bl04 and *B. longum* Bl05 in the fourth week of cold storage;

Similarly, assess the factors present in the apple and banana fibers that stimulate the viability of *L. acidophilus* L10, *B. lactis* Bl04, B94 and HN019.

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