UNIVERSITY OF SÃO PAULO

SCHOOL OF PHARMACEUTICAL SCIENCE

Graduate Program in Biochemical and Pharmaceutical Technology

Food Technology Area

Impact of the incorporation of probiotic strains and fruit by-products in a fermented synbiotic soy product and on the composition and metabolic activity of the gut microbiota *in vitro*

Antonio Diogo Silva Vieira

Thesis presented for the Degree of Doctor in Sciences, Graduate Program in Biochemical and Pharmaceutical Technology, Concentration area of Food Technology

São Paulo

2018

ANTONIO DIOGO SILVA VIEIRA

Impact of the incorporation of probiotic strains and fruit by-products in a fermented synbiotic soy product and on the composition and metabolic activity of the gut microbiota *in vitro*

Corrected version of the thesis according to the resolution CoPGr 6018

Thesis presented for the Degree of Doctor in Sciences, Graduate Program in Biochemical and Pharmaceutical Technology, Concentration area of Food Technology Ph.D.

Food Technology Area

Advisor:
Full Professor Susana Marta Isay Saad

São Paulo
2018

Ficha Catalográfica elaborada eletronicamente pelo autor, utilizando o programa desenvolvido pela Seção Técnica de Informática do ICMC/USP e adaptado para a Divisão de Biblioteca e Documentação do Conjunto das Químicas da USP

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

Vieira, Antonio Diogo Silva
V657i Impact of the incorporation of probiotic strains and fruit by-products in a fermented synbiotic soy product and on the composition and metabolic activity of the gut microbiota in vitro / Antonio Diogo Silva Vieira. - São Paulo, 2018.
190 p.

Tese (doutorado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Tecnologia Bioquímico-Farmacêutica.
Orientador: Saad, Susana Marta Isay

1. Food Technology. 2. Functional Foods. 3. Industrial by-products. 4. Gut Microbiota. 5. Probiotic and Prebiotic. I. T. II. Saad, Susana Marta Isay, orientador.

VIEIRA, A.D.S. **Impact of the incorporation of probiotic strains and fruit by-products in fermented synbiotic soy product and on the composition and metabolic activity of the gut microbiota *in vitro*.** 2018. 190 pags. Thesis (Doctorate in Biochemical-Pharmaceutical Technology, Concentration area: Food Technology) – School of Pharmaceutical Science, University of São Paulo, São Paulo, 2018.

Evaluated by:

Judging Committee of
thesis to obtain the degree of Doctor in Science:

_____

Full Professor Dr Susana Marta Isay Saad
School of Pharmaceutical Science - USP
Advisor/Principal

_____

Assistant Professor Dr. Roberta Claro da Silva
College of Agriculture and Environmental Sciences (CAES)
North Carolina A&T State University

_____

Dr. Katia Sivieri
School of Pharmaceutical Science - UNESP

_____

Dr. Carla Taddei de Castro Neves
School of Pharmaceutical Science - USP

_____

Full Professor Dr. Bernadette Dora Gombossy de Melo Franco
School of Pharmaceutical Science - USP

I dedicate this thesis to my husband, João Amauri B. Vieira (Junior) and my family, who supported me, dedication and patience throughout the journey to obtain my doctorate. *In memoria* of my grandfather Eduardo Vieira Neto.

AGRADECIMENTOS

A Deus, sempre, pela vida, pela minha família, esposo e amigos, por sempre está comigo, em meu coração, e sempre me ter em seus braços principalmente nos momentos mais difíceis.

Ao meu esposo João Amauri (Junior), pelo apoio, amor e compreensão durante toda a minha caminhada para a obtenção do título de doutor e na vida.

Aos meus pais José e Evenir, irmão, Dyego e meus lindos sobrinhos Paulo Eduardo e o Joaquim, e toda a minha família, pelo amor, carinho, compreensão, pelo apoio e palavras de sabedora, principalmente nos momentos em que me encontro perdido, sempre posso contar com eles.

À minha orientadora Profª Dra. Susana Marta Isay Saad, por acreditar no meu potencial e que conseguiria crescer como pessoa e profissional durante o desenvolvimento do projeto de doutorado.

Ao meu supervisor do estágio no exterior Profº Dr. Koen Venema, por ter me recebido em seu laboratório na Maastricht University (Venlo, Holanda), pelo apoio, ensinamentos, durante o período em que estive na Holanda e após o desenvolvimento do meu projeto de pesquisa no exterior.

Às minhas grandes amigas Dra. Vanessa Biscola e Dra. Roberta Claro pela colaboração nos trabalhos, companheirismo e amizade.

À Dra. Raquel Bedani, pela colaboração durante a redação do projeto, durante a realização do meu trabalho, e no fim a redação dos artigos, pelas ideias e conhecimentos trocados durante todo o desenvolvimento do meu trabalho.

A meus queridos amigos e colegas do grupo de pesquisa, Clara, Dr. Douglas Xavier e Carolina, pela agradável convivência, Igor e Luiz, pela grande ajuda durante as minhas análises sensoriais, em especial, a Dra. Natalia Matias, Dra. Mayra Garcia, Dra. Marina Padilha e Marcela minhas companheiras de todas as horas.

A Sra. Isabel Bossi, pelo auxílio nas questões financeiras e organização de documentos das prestações de conta FAPESP e CNPq, além da amizade.

A minha co-orientanda de Iniciação Científica Bruna Tomas, peja ajuda durante a realização das primeiras etapas do meu projeto de doutorado.

Aos membros da FBT, Alexandre Mariane, Ivani, Nilton (técnicos), Rosi, Tania e Elza (Secretaria) e a Tatiana do Setor de Convênios da FCF/USP.

Ao pessoal do Laboratório de Microbiologia de Alimentos, Profª Dra. Bernadette Franco, Profª Dra. Mariza Landgraf, Profº Dr. Uelinton Pinto, Profº Dr. Christian Hoffmann, Katia Leane, Lucia, Daniel, Raquel e Dra. Daniela Maffei.

A Profª Dra. Ines Genovese, Tatiana e técnica Rosa da FBA/USP, e a Profª Dra. Juliana Ract pelo auxílio na realização de algumas análises do meu projeto de doutorado.

Ao pessoal do Laboratório da Maastricht University, da Brigthlab (Venlo, Holanda) e Laboratórios de Microbiologia e Biologia molecular da Wageningen University (Wageningen, Holanda), Rob, Soyna e Bart (Venlo); Ineke e Steave (Wageningen).

Aos meus colegas de Laboratório da Maastricht University, Maria Nieto, Angela e Long, em especial as minhas queridas amigas Maria Cuebas e Carlota Bussolo pela amizade e ajuda durante a realização dos meus experimentos na Maastricht University.

Aos Professores Dr. Erwin Zoetendal e Dr. Hauke Smitd da Wageningen University, por me receberem em seus laboratórios durante parte do meu trabalho de estágio no exterior e pela colaboração do nos trabalhos posteriores.

As instituições de ensino superior Universidade de São Paulo, Maastricht University e Wageningen University, onde realizei as atividades do meu doutorado.

Aos membros da Banca de Qualificação de Doutorado Profª Gisele Monteiro de Sousa (FBT/USP), Profº Uelington Manoel Pinto (FBA/USP) e Profª Cynthia Jurkiewicz Kunigk (Instituto Mauá) pela avaliação e contribuição no presente trabalho.

Às agências de fomento, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Processo CNPq: 164341/2013-7) pelos primeiros 5 meses de bolsa de doutorado e à Fundação de Pesquisa do Estado de São Paulo (**FAPESP**), pelas bolsas de doutorado (Processo FAPESP: 2013/19346-4) e bolsa de pesquisa no exterior (Processo FAPESP BEPE: 2016/07940-7).

Ao projeto temático de pesquisa BIOSYN (FAPESP, Processo: 2013/50506-8), de coordenação da Profª Susana Saad, pelo auxílio financeiro para realização do meu projeto de doutorado.

Às indústrias de processamentos de frutas Cutrale® (Araraquara, São Paulo, Brasil), Ricaeli Alimentos (Cambreuva, São Paulo, Brasil), De Marchi (Jundiaí, São Paulo, Brasil) e Sítio do Belo (Paraibuna, São Paulo, Brasil) pelo fornecimento dos resíduos do processamento de frutas, assim como a Unidade de Desenvolvimento e Produção de Derivados de Soja (UNISOJA, UNESP, Araraquara) pelo fornecimento dos resíduos do processamento de soja (okara).

Às empresas AgarGel® (São Paulo) em nome de Camila Cavalcante, Labonathus Biotecnologia Internacional Ltda. Em nome de Bianca Dalonso e Carolina Moura pela doação de ingredientes empregados nas formulações das bebidas fermentadas de soja.

A Chr. Hansen e a Dupont pela doação das cepas probióticas e starter utilizadas no presente estudo.

As Profª Maria Angélica Barone (Diretora da FATEC) e Profª Taís Moreira (Coordenadora do Curso de Alimentos) da FATEC Oswaldo Cruz, pela oportunidade de ministrar aulas no Curso de Tecnologia de Alimentos Industrializados.

A todos que direta ou indiretamente contribuíram para a realização deste trabalho.

ACKNOWLEDGEMENTS

I wish to thanks:

To God, always, for my life, for my family, husband, and friends, and for always be with me, in my heart, and for always hold me in His arms, especially in the most difficult moments.

To my husband João Amauri (Junior), for the support, love and understanding during my journey to obtain the Ph.D. degree, and in the life.

To my parents José (Matenga) and Evenir, my brother Dyego, and my sweet nephews Paulo Eduardo and Joaquim, and my whole family, for love, affection, understanding, support and words of wisdom, especially in the moments, when I feel lost, I always can rely on them.

To my advisor Prof. Dr. Susana Marta Isay Saad, for believing in my potential, and that I could grow personally and professionally during the development of my Ph.D. project.

To my advisor abroad, Prof. Dr. Koen Venema, for having received me in his laboratory at Maastricht University (Venlo, The Netherlands), for the support and teachings during the time I was in the Netherlands, and after that in the preparation of articles for future publications.

To my great friends Dr. Vanessa Biscola and Dr. Roberta Claro, for the collaboration in the works, companionship and friendship.

To Dr. Raquel Bedani, for the collaboration during the writing of this project and accomplishment of my work, and in the end, helping in the writing of articles. Moreover, for the ideas and knowledge exchanged throughout the development of my Ph.D.

To my dear friends and colleagues from the research group, Clara, Dr. Douglas Xavier, and Carolina, for the pleasant coexistence, Igor and Luiz, for the immense help during my sensorial analysis, and especially to Dr. Natalia Matias, Dr. Mayra Garcia, Dr. Marina Padilha and Marcela my companions of all hours.

To Mrs. Isabel Bossi, for the assistance in financial matters related to FAPESP and CNPq fellowships, moreover to her friendship.

To the undergraduate student Bruna Tomaz, my co-mentee, for helping me during the first stages of my Ph.D. project.

To the staffs of the FBT, Alexandre Maria, Ivani, Nilton (technicians), Rosi, Tania, and Elza (Secretariat), and Tatiana from the FCF/USP's Agreements Sector.

To the staffs of the Food Microbiology Laboratory (FBA/USP): Professors Dr. Bernadette Franco, Dr. Mariza Landgraf, Dr. Uelinton Manoel Pinto, and Dr. Christian Hoffmann, technicians Katia Leane and Lucia, Ph.D. students Daniel and Raquel, and Dr. Daniela Maffei.

To Prof. Dr. Ines Genovese, Tatiana and technician's Rosa from FBA/USP, and Prof. Dr. Juliana Ract for the assistance in performing some analysis of my PhD project.

To the staffs of the Maastricht University Laboratory, Brigthlab (Venlo, The Netherlands) and Microbiology and Molecular Biology Laboratories of Wageningen University (Wageningen, The Netherlands): Rob, Soyna and Bart (Venlo); Ineke and Steave (Wageningen).

To my laboratory colleagues at Maastricht University, Maria Nieto, Angela and Long, and especially to my dear friends Maria Cuebas and Carlota Bussolo, for the friendship and help during my experiments at Maastricht University.

To Professors Dr. Erwin Zoetendal and Dr. Hauke Smitd of Wageningen University, for receiving me in their laboratories during part of my work abroad, and for the collaboration of the later works.

To institutions of education and research University of São Paulo, Maastricht University and Wageningen University, where I conducted my Ph.D. activities.

To the members of the judging committee of Ph.D. qualification exam, Professors Dr. Gisele Monteiro de Sousa (FBT/USP), Dr. Uelington Manoel Pinto (FBA/USP) and Dr Cynthia Jurkiewicz Kunigk (Mauá Institute), for evaluation and contribution in the present work.

To the research development agencies, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Process CNPq: 164341/2013-7) for the first 5 months of the Ph.D. fellowship and to the Fundação de Pesquisa do Estado de São Paulo (**FAPESP**) for the Ph.D. fellowship (FAPESP Process: 2013/19346-4), and research fellowship abroad (FAPESP BEPE Process: 2016/07940-7).

To the thematic research project BIOSYN (FAPESP, Process: 2013/50506-8), coordinated by Dr. Susana Saad, for the financial assistance to carry out my Ph.D. project.

To the fruit processing industries Cutrale® (Araraquara, São Paulo, Brazil), Ricaeli Alimentos (Cambreuva, São Paulo, Brazil), De Marchi (Jundiaí, São Paulo, Brazil), and Sítio do Belo (Paraibuna, (UNISOJA, UNESP, Araraquara), for supplying of the by-products.

To Camila Cavalcante (AgarGel®, São Paulo), and Bianca Dalonso and Carolina Moura (Labonathus Biotecnologia Internacional Ltda), for the supply of ingredients used in the formulations of fermented soy beverages.

To Chr. Hansen and DuPont, for the supply of the probiotic and starter strains used in the present study.

To the lecturers Maria Angélica Barone (Principal of FATEC) and Taís Moreira (Coordinator of the Food Technology graduation course) of FATEC Oswaldo Cruz, for the opportunity to teach classes in the Industrialized Food Technology graduation course.

To all who directly or indirectly contributed to the accomplishment of this work.

“If you don't love yourself, how in the hell you gonna love somebody else?”

RuPaul Andre Charles (RuPaul)

VIEIRA, A.D.S. **Impacto da incorporação de cepas probióticas e de subprodutos de frutas em um produto fermentado de soja simbiótico e sobre a composição e a atividade metabólica da microbiota intestinal humana *in vitro.*** 190 pag. Tese (Doutorado em Tecnologia Bioquímico-Farmacêutica, Area de concentração: Tecnologia de Alimentos) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, 2018.

RESUMO

O presente trabalho visou desenvolver uma bebida fermentada de soja adicionada de resíduos de frutas e suplementada com cepas probióticas e avaliar o impacto desse produto sobre a composição e a atividade metabólica da microbiota intestinal humana, utilizando um modelo de simulação *in vitro* das condições intestinais (TIM-2). Para tanto, o presente trabalho foi dividido em três etapas. A etapa I foi baseada na obtenção, processamento e caracterização físico-química, microbiológica e funcional de subprodutos de frutas (acerola, laranja, manga e maracujá) e soja (okara), bem como da farinha de amaranto. Adicionalmente, a capacidade de utilização desses subprodutos vegetais e da farinha de amaranto por cepas probióticas e não probióticas foi avaliada. Os resultados mostraram que o subproduto de acerola apresentou o maior conteúdo de fibras alimentares totais (48,46 g/100 g) dentre os subprodutos testados, bem como a farinha de amaranto. Os subprodutos de laranja e maracujá foram os substratos que mais promoveram a multiplicação das populações bacterianas, incluindo das cepas de *Escherichia coli* e *Clostridium perfringens*. Por outro lado, o subproduto de acerola foi o substrato que apresentou a maior seletividade para bactérias benéficas. Ainda nessa etapa, dez cepas probióticas (sete lactobacilos e três bifidobacterias) e três cepas *starter* (*Streptococcus thermophilus*) foram testadas quanto à sua capacidade de desconjugação de sais biliares e atividade proteolítica frente às proteínas do leite e da soja. Os resultados revelaram que nenhuma cepa testada apresentou capacidade de proteólise das proteínas do leite e da soja. Adicionalmente, as cepas probióticas *Lactobacillus acidophilus* LA-5 e *Bifidobacterium longum* BB-46 desconjugaram a maior quantidade de ácidos biliares testados e as cepas de *S. thermophilus* testadas não apresentaram capacidade de desconjugação de sais biliares. Após a análise dos resultados da etapa I, o resíduo de acerola (ABP) e as cepas probióticas LA-5 e BB-46 foram selecionadas para dar continuidade à etapa II do estudo(desenvolvimento de uma bebida fermentada a base de soja). Para esse fim, foi utilizado um delineamento experimental do tipo fatorial 2^3, totalizando 8 ensaios com três repetições de cada, e foram avaliados os efeitos das cepas probióticas e do subproduto de acerola sobre as características físico-químicas, microbiológicas e sensoriais dessas bebidas fermentadas de soja. Paralelamente, foram realizadas análises da sobrevivência das cepas probióticas frente às condições gastrintestinais simuladas *in vitro* nas bebidas fermentadas de soja (FSB). Os resultados mostraram que a presença de BB-46 e ABP afetaram negativamente a aceitabilidade sensorial das FSB. O ABP também levou a diferenças significativas no perfil de textura das FSB ($P<0,05$). As populações das cepas probióticas nas diferentes formulações de FSB variaram de 7,0 a 8,2 log de UFC equivalente/mL durante os 28 dias de armazenamento (4 ºC) e a co-cultura (LA-5+BB-46) e o ABP não afetaram ($P>0,05$) a viabilidade de ambos os microrganismos. No entanto, ABP aumentou significativamente a sobrevivência de BB-46 frente às condições gastrintestinais sumuladas *in vitro*. Para a etapa III do presente estudo, um delineamento experimental fatorial 2^2 foi realizado. Para a avaliação do impacto dessas FSB sobre a composição e atividade metabólica da microbiota intestinal de humanos eutróficos e obesos, foi utilizado um modelo *in vitro* TIM-2 na *Maastricht University* (Venlo, Holanda), que simula as condições normais do lúmen do cólon proximal, com todos os parâmetros controlados por um computador. Amostras foram coletadas do TIM-2 para a quantificação dos microrganismos probióticos (LA-5 e BB-46), *Lactobacillus* spp., *Bifidobacterium* spp. e bactérias totais, utilizando o método de PCR quantitativo (qPCR), e

o perfil da microbiota intestinal foi determinado utilizando Next-Generation Sequencing (NGS) Illumina Mysec. A concentração de ácidos graxos de cadeia curta e de cadeia ramificada e lactato produzidos pelas diferentes microbiotas durante a fermentação no TIM-2 também foi determinada. Os resultados mostraram que a microbiota de humanos eutróficos apresentou uma alta produção de acetato e lactato em comparação com a microbiota de obesos. Reduções significativas das populações de *Bifidobacterium* na microbiota de eutróficos foram observadas entre 0 e 48 h de ensaio para todas as refeições experimentais, exceto para a refeição que apresentou a combinação probiótica (LA-5 e BB-46) e a suplementação com ABP, que apresentou aumento de *Bifidobacterium* e *Lactobacillus* totais durante todo o período de análise para ambas as microbiotas testadas. As FSB suplementadas com ABP apresentaram os melhores resultados em relação à modulação da microbiota de humanos obesos, com o aumento *Bifidobacterium* spp. e *Lactobacillus* spp. Adicionalmente, após 48 horas de intervenção no TIM-2, a microbiota de obesos foi aparentemente similar à microbiota de eutróficos, mostrando uma modulação benéfica dessa microbiota. Os resultados sugerem que as bebidas fermentadas de soja suplementadas com o subproduto de acerola e cepas probióticas podem apresentar efeitos benéficos à saúde. No entanto, estudos clínicos são necessários para complementar e confirmar os resultados observados nos ensaios *in vitro*.

Palavras chave: Probioticos e prebioticos, sub-produto de frutas, microbiota intestinal, bebida fermentada de soja, acerola

VIEIRA, A.D.S. **Impact of the incorporation of probiotic strains and fruit by-products in a fermented synbiotic soy product and on the composition and metabolic activity of the gut microbiota *in vitro.*** 2018. 190 pags. Thesis (Doctorate in Biochemical-Pharmaceutical Technology, Concentration area: Food Technology) – School of Pharmaceutical Science, University of São Paulo, São Paulo, 2018.

ABSTRACT

The present study aimed to develop a fermented soy beverage containing fruit by-products and probiotics and to evaluate the impact of this product on the composition and metabolic activity of the human intestinal microbiota using an *in vitro* simulation model of the intestinal conditions (TIM-2). Therefore, the present study was divided into three stages. Stage I was based on obtaining, processing and physical-chemical, microbiological and functional characterization of fruit by-products (acerola, orange, mango, and passion fruit) and soybean (okara), as well as amaranth flour. Additionally, the ability to use these vegetable by-products and amaranth flour by probiotic and non-probiotic strains was evaluated. The results showed that the acerola by-product presented the highest dietary fibre content (48.46 g/100 g) among the by-products tested, as well as amaranth flour. Orange and passion fruit by-products were the substrates that most promoted the growth of bacterial populations, including strains of *Escherichia coli* and *Clostridium perfringens*. On the other hand, the acerola by-product was the substrate that showed the highest selectivity for beneficial bacteria. Also, in this stage, ten probiotic strains (seven lactobacilli and three bifidobacteria) and three starter strains (*Streptococcus thermophilus*) were tested for their ability to deconjugate bile salts and for proteolytic activity against milk and soy proteins. The results showed that none of the tested strain showed proteolytic ability against milk and soybean proteins. In addition, the probiotic strains *Lactobacillus acidophilus* LA-5 and *Bifidobacterium longum* BB-46 deconjugated more types of bile acids tested, and the strains of *S. thermophilus* tested showed no ability to deconjugate bile salts. Next, the acerola by-product (ABP) and the probiotic strains LA-5 and BB-46 were selected to continue stage II of the study (development of a fermented soy beverage). For this purpose, a 2^3 factorial design was used, in a total of 8 trials with three replicates of each one, and the effects of the probiotic strains and the acerola by-product on the physical-chemical, microbiological, and sensory characteristics of these fermented soy beverages were evaluated. At the same time, probiotic viability and survival under *in vitro* gastrointestinal (GI) simulated conditions were evaluated in fermented soy beverage (FSB). The results showed that the presence of BB-46 and ABP affected the sensory acceptability of FSB negatively. ABP also led to significant differences in the texture profile of the FSB ($P<0.05$). Populations of probiotic strains ranged from 7.0 to 8.2 log CFU equivalent/mL during 28 days of refrigerated storage (4 ° C) of FBS, and the co-culture (LA-5+BB-46) and the ABP did not affect the viability of both microorganisms significantly (P> 0.05). However, ABP increased the survival of BB-46 under *in vitro* simulated GI conditions significantly. For stage III, a 2^2 experimental design was performed. To evaluate the impact of these FBS on the composition and metabolic activity of the intestinal microbiota of lean and obese humans, a validated *in vitro* model called TIM-2 was used, available at the Maastricht University (Venlo, The Netherlands), which simulates normal conditions of the lumen of the proximal colon, with all parameters controlled by a computer. Samples were collected from TIM-2 to quantify probiotic microorganisms (LA-5 and BB-46), *Lactobacillus* spp., *Bifidobacterium* spp., and total bacteria, using the quantitative PCR method (qPCR) and the intestinal microbiota profile was determined using an Illumina Mysec Next Generation Sequencing (NGS) method. Concentrations of short-chain fatty acids and branched-chain fatty acids and lactate produced by the different microbiotas during fermentation in TIM-2 were also determined. The results showed that the lean microbiota presented the high production of acetate and lactate than the microbiota of obese individuals.

Significant reductions in *Bifidobacterium* populations in the lean microbiota were observed at 0 and 48 h of an assay for all experimental meals, except for the meal that had the probiotic combination (LA-5 and BB-46) and the ABP supplementation, which showed an increased total *Bifidobacterium* and *Lactobacillus* populations throughout the experimental period for both microbiotas tested. The FSB supplemented with ABP presented the best characteristics regarding the modulation of the obese microbiota, with an increase in *Bifidobacterium* spp. and *Lactobacillus* spp. Additionally, after 48 hours of intervention in TIM-2, the obese microbiota was apparently similar to the lean microbiota, showing a beneficial modulation of this microbiota. The results suggest that the fermented soy beverage supplemented with the acerola by-product and the probiotic strains may present beneficial health effects. However, clinical studies are required to complement and confirm the results observed in the *in vitro* assays.

Keywords: Probiotic and Prebiotic, fruit by-products, gut microbiota, fermented soy beverages, acerola

ILUSTRATION LIST

TABLES LIST

Content

Chapter 1

General Introduction:
Fruit and vegetable by-products and their application in probiotic food products

1. PROJECT JUSTIFICATION

Currently, Brazil has become a major exporter of agricultural products, being one of the largest exporters of *in nature* and processed fruit and grains, with a surplus of USD 78.6 billion in 2013 (OECD/FAO, 2015). The production and processing of vegetable products generate a large amount of waste and by-products (peels, seeds, leaves, stalks, and pulps), most of which are incinerated for the production of energy in the industries and used in composting as feed or discarded indiscriminately in the environment. Nonetheless, these by-products can be rich in bioactive compounds and, therefore, could be used for human nutrition purposes. However, these by-products are lost and discarded due to a lack of information and/or technologies that can take advantage of and add value to such wastes.

Several studies have shown the nutritional and functional properties of various industrial by-products of fruits and other agricultural products, since these by-products may present, for example, a high concentration of dietary fibres (AJILA; LEELAVATHI; RAO, 2008; AJILA et al, 2010; BENÍTEZ et al., 2012), phenolic compounds, antioxidants (AJILA; LEELAVATHI; RAO, 2008; BARROS; FERREIRA; GENOVESE, 2012; DUZZIONI et al., 2013), vitamins, and minerals (VIUDA-MARTOS et al., 2009; DUZZIONI et al., 2013).

In addition to the development of new technologies for the use of vegetable by-products in human nutrition, the best alternatives for the administration and inclusion of these residues in the diet should be evaluated. An alternative for the application of these by-products is their incorporation in a fermented soy product.

Soybean is the food that offers the greatest possibilities for the development of functional products in Brazil, due to its great production in the country, its nutritional and functional value with several beneficial health effects related to its consumption, such as reduction of serum cholesterol, reducing the risk of developing cancer and osteoporosis, among other health effects.

The combination of the functional properties of the fruit by-products and the water-soluble soy extract with the presence of probiotic microorganisms and prebiotic ingredients results in a fermented multifunctional soy product with great potential for modulation and modification of the human intestinal microbiota. In addition to the functional potential generated by the use of by-products of fruit processing, it is important to mention that the present project has a sustainable appeal, since the use of these by-products adds value to a product that would be discarded by the industry, with significant damages to the environment. In this sense, the incorporation of fruit by-product could also represent a promising alternative to confer technological, sensorial and functional characteristics to fermented soy products.

2. FRUIT AND VEGETABLE BY-PRODUCTS AND THEIR APPLICATION IN PROBIOTIC FOOD PRODUCTS

2.1. Brazilian agriculture, industrialization, and sustainability

In the 1970's, Brazil was dependent on imports of several basic foodstuffs, which has been changing in the last four decades, with outstanding performance in the innovation and production agriculture. Currently, the agriculture sector plays an important role in supporting Brazil's economic performance. In the last years, the increase in the area of fruit-growing, accounts for 13% of the value of national agriculture production, contributing approximately US$ 12.2 billion in the formation of the national Gross Domestic Product (GDP). Additionally, in 2010, the average productivity of grain crops achieved 3,173 kilos per hectare, representing a jump of 774% in the production (OECD/FAO, 2015; LOPES & CONTINI, 2012). Nonetheless, the success of Brazil's agribusiness has been correlated with widespread destruction of natural resources and Brazilian ecosystems (MARTINELLI et al., 2010).

Brazil, like no other country in the world, has the potential to improve economic and sustainability of the new bio-industry. Changes in the Brazilian vision about agricultural policy have sought to develop sustainable alternatives for the country's agricultural development. Several credit programmes for the family farm segment with an environmental focus have been develop in the last years. The agriculture of the future should be marked by concepts, multifunctional methods and applications, far beyond the conventional agriculture, dedicated to food, fibres, and energy production (OECD/FAO, 2015; LOPES & CONTINI, 2012).

Due to the changes of the socioeconomic situation in Brazil, the sales of fruits and vegetables with some degree of processing, associated with the improvement of the products' quality offered and their growing presence in the networks of distribution, has increased the consumption of processed fruits and vegetables compared to the fresh fruit and vegetables consumption (AGROCLUSTER RIBATEJO, 2015). Worldwide, the fruit processing industries generate more than 0.5 billion tonnes of by-products and waste (BANERJEE et al., 2017) with great potential utilization after their processing. Vegetables by-products can be considered an abundant source of bioactive compounds, such as dietary fibre, phenolic compound, antioxidants, polyunsaturated fatty acids, vitamins, and minerals. In this context, industrial ecology concepts as biomimetic approach to the design of products (cradle to cradle) and circular economy are considered main principles for ecology innovation, pointing at "zero waste", in which residue are used as raw material and ingredients to be employed in new products and processes (GALANAKIS, 2013; KOWALSKA et al., 2017; MIRABELLA; CASTELLANI; SALA, 2014).

2.2. Fruit and vegetables by-products as sources of bioactive compounds and ingredients

Several studies have reported the versatility in the use of various fruit, vegetable, and cereal by-products in the development of foods with functional appeal and/or potential health benefits and well-being. As previously described, these by-products are rich in bioactive compounds which may be used for various purposes. The bioactive compounds contained in the matrix of such by-products may be higher than those found in the juice or pulp. Studies have shown that the peel and seeds of certain fruits may have a higher antioxidant activity than the pulp (DUZZIONI et al., 2013) and a large amount of dietary fibre in its soluble and insoluble forms (O'SHEA; ARENDT; GALLAGHER, 2012).

Considering the technological and microbiological attributes of vegetable by-products in meat products, FERNÁNDEZ-GINÉS et al. (2003) reported an improvement of nutritional characteristics of Bolongna sausage to which citrus fibre was added, with decreased levels of residual nitrite, as well as a delay in the oxidation process. A study conducted by SAYA-BARBERÉ et al. (2012) demonstrated that the addition of orange fibre and *Lactobacillus casei* CECT 475 in "*Longaniza de Pascua*" accelerated the pH decrease, reduced the counts of enterobacteria and residual nitrite compared to the other sausages. Additionally, the authors reported that sausage with orange fibre and *L. casei* strains presented the best sensory scores. In a study of the antifungal activity of polyphenols from olive mill wastewater, CHAVEZ-LÓPEZ et al. (2015) showed a microbiological protection effect, with the reduction or elimination of undesired fungi growth on the surface of dry fermented sausages, without changes in sensory characteristics.

Fruit and vegetables industrial by-products are a promising vehicle for the nutrients of bakery and pasta products and may improve their health and technological properties (MARTINS; PINHO; FERREIRA, 2017). AJILA et al. (2010) noted that the addition of mango peel powder in macaroni increased the polyphenol, carotenoid, and dietary fibre contents without changing the nutritional quality. Moreover, the authors reported that the resulting product presented a good sensorial acceptability. ESHAK (2016) described that partial replacement of wheat flour by banana peel flour increased up to 2% the protein and dietary fibre contents of bread, which showed the higher content of minerals like K, Na, Ca, Fe, Mg, and Zn, besides presenting a good sensorial acceptability. AJILA, LEELAVATHI, and RAO (2008) reported that the incorporation of 20% of mango peel flour in biscuits increased from 6.5 to 20.7% the dietary fibre content with a high proportion of soluble dietary fibre and there was an improvement of polyphenols levels from 0.54 to 4.5 mg/g of biscuit with mango peel flour. In

a study employing potato peel powder, BEN JEDDOU et al. (2017) demonstrated that potato peel powder improved the nutritional and the technological stability of cakes, with a reduction in hardness, an improved appearance of cakes, as well as an increased acceptability by the consumer panel.

The health benefits related to a decreased risk of cardiovascular disease have been attributed to the consumption of bread with grape by-product by MILDNER-SZKUDLARZ and BAJERSKA (2013). These researchers described a reduction of the negative impact of a high-cholesterol/cholic acid diet, lowering of serum total cholesterol and LDL-cholesterol, lipid peroxidation, glucose and leptin levels in rats that consumed bread supplemented with extract of grape peel and grape peel dried powder, which was rich in dietary fibre and antioxidants polyphenols.

Besides the bakery and meat products, fruit and vegetable by-products have been employed in dairy and vegetable "milk-like" products. Supplementation of yoghurts with apple, banana, and passion-fruit by-products increased the probiotic viability and fatty acid profiles with enhanced the conjugated linoleic acid (CLA) contents, as shown by ESPÍRITO SANTO et al. (2012). SENDRA et al. (2008) observed that the supplementation with citrus fibre enhanced the viability and survival of probiotic bacteria in fermented milk. Using olive and grape pomace phenolic extract in order to obtain a probiotic yoghurt fortified with natural antioxidants, therefore presenting a high biological value, ALIAKBARIAN et al. (2015) reported no interference of phenolic extracts in the yoghurts production and that a high content of phenolic compounds was maintained since, after 7 days of storage, the concentration of phenolic compounds in yoghurts was similar to that found in a spoon of olive oil. BEDANI et al. (2015) observed a reduction of LDL-cholesterol and LDL-cholesterol/HDL-cholesterol ratio in normocholesterolemic men that consumed a synbiotic fermented soy product with inulin and okara (soy by-product) for 8 weeks. In order to evaluate the effect of fruit and soybean by-product and amaranth flour on folate production by starter and probiotic strains, ALBUQUERQUE et al. (2016) observed that orange by-product was the substrate that presented the best production of folate by the tested probiotic and starter strains, while the presence of a higher initial content of folate in okara inhibited the production of folate. Moreover, the authors verified that folate production is not only strain-dependent but also influenced by the incorporation of different substrates in the growth media. In a more recent study, ALBUQUERQUE et al. (2017) described that the supplementation of fermented soymilk with passion-fruit by-product powder and fructo-oligossacharide (FOS) increased the

production of folate by *Streptococcus thermophilus* strains alone or in co-culture with lactobacilli strains.

2.3. Health benefits of functional probiotic, prebiotic, and synbiotic foods

The health and nutrition paradigms have changed significantly. In the last years, foods are not seen as only vehicles of nutrients for the growth and good development of the body, but also as a new way to optimize the health and well-being (AMERICAN DIETETIC ASSOCIATION, 2009). Advances in the gut microbiota studies are the main reasons for the growth of the functional food area since the gut microbiota is intimately related to human health and disease (MITSUOKA, 2014). Functional foods may be bioactive compounds present in natural food (e.g. essential fatty acids n-3 and n-6, vitamins, phenolic compounds, and other natural food bioactive compounds) or may contain one or more specific components (e.g. probiotic, prebiotic, synbiotic, bioactive peptides, dietary fibre, and other compound), which have beneficial influence on the host's health and well-being. It is important to emphasize that functional food does not necessarily induce a health benefit in all individuals of the population, since various factors such as environment, dietary habits, as well as genetic, biological, and metabolic factors are involved in the success or failure of a functional food health effect (TUR & BIBILONI, 2016).

Probiotic food products may be regarded as functional foods because they confer more health benefits than traditional foods do. The demand for functional probiotic food is progressively increasing as the consumers have become more aware of the impact of food on health. At the moment, probiotics are emerging as a promising category of food supplement worldwide (BEGUM et al., 2017). Probiotic are defined as “live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host” (HILL et al., 2014). Strains belonging mainly to the genera *Lactobacillus* and *Bifidobacterium* are the ones most explored as probiotics by the food industry (SÁNCHEZ et al., 2013). Some probiotic strains belong to the bacteria genera *Leuconostoc, Pediococcus, Enterococcus, Streptococcus, Bacillus,* and *Escherichia*, as well as yeasts belonging to the genera *Saccharomyces* (MARTINEZ; BEDANI; SAAD 2015; SÁNCHEZ et al., 2013; TAMANG et al., 2016).

Some of the health benefits attributed to the consumption of probiotics should be highlighted as follows: the improvement in the lactose intolerance symptoms; the control in the inflammatory bowel disease and irritable bowel syndrome (IBS) symptoms; beneficial changes in the composition and metabolic activity of the gut microbiota, with the colonization and inhibition of endogenous and exogenous pathogens; reducing the risk of colorectal cancer with

the production of anti-carcinogenic compounds, inhibition of cell proliferation, and induction of apoptosis in cancer cells; decreases in serum cholesterol levels and reducing the risk of cardiovascular diseases; improvement of the innate immunity; decrease in food allergy in infants; treatment and prevention of intestinal, respiratory tract and female urogenital infections; treatment of *Helicobacter pylori* gastric infection; reduction in the number of cariogenic streptococci in saliva and dental plaque; reduction of gingivitis and oral candidiasis infection, among others (BEGUM et al., 2017; MARTINEZ; BEDANI; SAAD, 2015; REIS et al., 2017). Nonetheless, the development of functional food products with appropriate probiotic dose at the time of consumption is a limiting factor, since several variables may affect the probiotic viability and survival during the food processing and storage (TRIPATHI; GIRI, 2014). In addition, the probiotic dose-response is influenced by several factors, including health endpoint, probiotic strain, delivery matrix, and administration form. These variables make it difficult to generalize one optimal concentration to obtain a probiotic effect (OUWEHAND, 2017). Therefore, maintenance of the viability of probiotic bacteria in the food matrix has been a prerequisite for ensuring its effect on the host health (ZACARIAS et al., 2011). SOHN and UNDERWOOD (2017) pointed out that innovative methods of probiotic delivery should be explored since nowadays probiotic products are limited to the microorganism's resistance to the gastrointestinal stress.

Strategies as the use of prebiotic ingredients may improve the survival and activity of probiotic strains during food storage, as well as during the transit through the gastrointestinal tract (GIT) (DONKOR et al., 2007). As happened with the probiotic definition, the ISAPP (International Scientific Association for Probiotics and Prebiotics) reviewed the definition and scope of prebiotic compounds. Nowadays, the definition of prebiotics which previously covered only non-digested carbohydrates selectively fermented by beneficial microbes conferring a health benefit to the host (GIBSON; ROBERFROID, 2008) was modified to "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (GIBSON et al., 2017). Currently, beyond some non-digested carbohydrates, the prebiotic definition contemplates other substance, as human milk oligosaccharides and polyunsaturated fatty acids, as well as phenolic and phytochemical compounds (GIBSON et al., 2017). The selective fermentation of prebiotic by beneficial bacteria and the production of some beneficial metabolic compounds as short-chain fatty acid (SCFA) is associated with improved satiety and weight loss, decrease in systemic inflammation, increase of the intestinal barrier function, and enhanced glucose and fat metabolism (O'CONNOR et al., 2017). Besides the microbiota modulation, studies have reported beneficial prebiotic health effects on stress, with the

reduction of stress-induced corticosterone release and modification of specific gene expression in the hippocampus and hypothalamus, thus exhibiting antidepressant and anxiolytic effects (BUROKAS et al., 2017).

A synbiotic food must contain both probiotic microorganisms and prebiotic ingredients. Currently, the definition of synbiotic includes two approaches: complementarity and synergy. In the complementary approach, the probiotic strain is chosen based on the beneficial effects on the host, independently of the prebiotic chosen, to selectively increase the concentration of beneficial microorganisms already present in the gastrointestinal tract. In the synergistic approach, the prebiotic compound is chosen specifically to stimulate the multiplication and the activity of the probiotic selected for addition in the product, thus contributing for its survival in the GIT (SU; HENRIKSSON; MITCHELL, 2007; KOLIDA & GIBSON, 2011). Synergic effects were reported by KOJIMA et al. (2016) when five lactobacilli strains isolated from the oral cavity were combined with arabinose, xylose, and xylitol since Lactobacillus strains isolated from dairy products did not show a significant effect on the human oral microbiota. The authors demonstrated that synbiotic combinations inhibited the growth of oral pathogens, as well as had an inhibitory effect on the *Streptococcus mutans* production of insoluble glucan. To evaluate the effects of the consumption of a synbiotic food by diabetic patients, ASEMI et al. (2014), using randomized control trials, demonstrated that the consumption of a synbiotic food for 6 weeks resulted in a significant effect on various metabolic parameters, among them decreased serum insulin levels, high sensitivity C-reactive protein (hs-CRP) levels, and total glutathione. Also, beneficial effects on insulin metabolism were verified by TAJADADI-EBRAHIMI et al. (2014) during 8 weeks of consumption of synbiotic bread by patients with type 2 diabetes mellitus. According to a systematic review published by FERNANDES et al. (2017), some studies in humans with overweight and/or obesity showed health beneficial effects from the consumption of prebiotic and synbiotic foods on inflammatory markers, with the reduction of hs-CRP, endotoxin, and interleukin-6 and/or tumor necrosis factor levels.

2.4. Gut microbiota and its effects on health

The human gastrointestinal tract hosts a complex set of microorganisms which form a specific microbiota to each person, including trillions of bacteria, archeas, virus, and some uni and/or multicellular eukaryotes. These microorganisms essentially cover the mucosal surface of the host and are collectively referred to as a microbiota (SEKIROV et al., 2010; SOMMER & BÄCKHED, 2013). The gut microbiota is diverse and dynamic in the first years of life,

playing an essential role in health and disease in later life. It tends to stabilize in childhood, having a great influence on the development of the digestive tract, immune system, and metabolic pathways (COLLADO et al., 2016; TANAKA & NAKAYAMA, 2017; MULLER et al., 2015; BACKHED et al., 2015).

Until recently it was believed that the gastrointestinal tract of the newborns was sterile and immediately colonized after birth (BIASUCCI et al., 2010). Nevertheless, recent studies have demonstrated the presence of microorganisms in the amniotic fluid, placenta, foetal membranes, umbilical cords, and meconium (AAGAARD et al., 2014; CHU et al., 2017; COLLADO et al., 2016; DIGIULIO et al., 2008; 2010; JIMÉNEZ et al., 2005; ROMAN et al., 2015; ROMERO et al., 2015), which may indicate that the colonisation of the intestinal microbiota begins in the uterus and not after birth (COLLADO et al., 2016; TANAKA & NAKAYAMA, 2017). A direct impact of prenatal microorganisms on foetal gut colonization was reported by COLLADO et al. (2016). These authors hypothesized that the process of maturation of a healthy immune system developed by the contact with gut microorganisms may begin already during foetal life. The gut colonization is not random and several changes may take place in the prevalent species, during the first month of life (DOGRA et al., 2015). MOLES et al. (2013) reported that the specific microbiota differs between spontaneously-released meconium and faeces after the first week of life. In a study with Swedish infants and their mothers, BÄCKHED et al. (2015) demonstrated that the infants born by caesarean-section had a less resemblance to their mothers' microbiota when compared with infants from vaginally birth. Additionally, the authors reported that nutrition had a great impact in the composition and function of the early microbiota, with an increase in the proportion of species of the genera *Roseburia*, *Clostridium,* and *Anaerostipes* for the infants who were not breastfed, while for the breastfed infants aged 12 months the beneficial genera *Bifidobacterium* and *Lactobacillus* dominated the gut microbiota.

Traditionally, studies on the gut microbiota were focused on analysing its role in human disease. However, recently, this research field has significantly increased, resulting in the publication of several reports that describe the wide impact of the intestinal microbiota on the host physiology. Thus, the traditional anthropocentric view of the gut microbiota as pathogenic and as an immunological threat has been substituted with an appreciation of its mainly beneficial influence on the human health (SOMMER & BÄCKHED, 2013). The development of molecular techniques as Next-Generation Sequence technologies (NGS) has represented a significant improvement in metagenomic research, leading to a substantial increase in the knowledge about the different microbiomes and their influence on the human

host and the gut microbiota. Therefore, the increased knowledge about the gut microbiome functions favours the development of new therapeutic strategies based on the microbiome manipulation (D'ANGENIO & SALVATORE, 2015).

Until now, the intestinal microbiota is considered as an "organ" that affects the host biology, generating energy from waste decomposition undigested in the small intestine (complex carbohydrates and proteins), resulting in fermentation products (short-chain fatty acids, ethanol, gases, vitamins, among others). The intestinal microbiota acts as a barrier against a number of pathogenic microorganisms, inhibiting their invasion by decreasing the permeability of the epithelium due to an increase in the expression of proteins occlusion zonules of enterocytes and to the regulation of the innate immunity by a receptor recognition of bacterial antigens (LI et al., 2016; TESTRO & VISVANATHAN, 2009). An important activity of the intestinal microbiota is the utilization of nutrients that are not completely hydrolysed by enzymes in the human gastrointestinal tract and are lost with the excretion (KIMURA et al, 2013).

In general, a high diversity in the microbiota is considered as an index of the health condition, while a decrease in the bacterial diversity has been connected with the development and progression of human diseases like obesity and colorectal cancer, as well as inflammatory and immunological diseases (AMBALAM et al., 2016; D'ANGENIO & SALVATORE, 2015; LI et al., 2016). Additionally, studies have shown that the gut microbiota can influence several neuropsychiatric disorders (BRUCE-KELLER; SALBAUM; BERTHOUD, 2018). KIM et al. (2018) showed that personality traits are correlated with the composition and diversity of the human gut microbiota. In addition, dysbiosis in the gut microbiota are closely related to Parkinson's disease (PARASHAR & UDAYABANU, 2017), autism spectrum disorders in children (KANG et al., 2018; LI & ZHOU, 2016; VUONG & HSIAO, 2017), anxiety and depressive disorders (BRUCE-KELLER; SALBAUM; BERTHOUD, 2018), as well as Alzheimer's disease (GUBIANI et al., 2017; MANCUSO & SANTANGELO, 2018).

In view of this, the gut microbiota modulation by probiotics and prebiotics has been pointed out as a promising alternative to prevent some diseases (BUTEL, 2014; DINAN & CRYAN, 2013). In a review, AMBALAM et al. (2016) reported several studies in which probiotics and prebiotics, alone or in combination (synbiotics), could modulate the immune system and the gut microbiota, thus preventing an inflammation process and colorectal cancer. BAGAROLLI et al. (2017) reported that probiotic strains modulated the gut microbiota in an *in vivo* model using DIO mice, reversing the obesity-related characteristics, inducing an increase in the hypothalamic insulin resistance. RASMUSSEN and HAMAKER (2017) showed

that prebiotic and dietary fibres, when fermented by the gut microbiota, resulted in the modulation of this intestinal microbiota and in the production of metabolic compounds with beneficial effects like short-chain fatty acids (SCFA), which could reduce the inflammation process in inflammatory bowel disease.

2.5. Soybean functional probiotic and prebiotic products

Currently, the use of beneficial bacteria as probiotic strains and also prebiotic ingredients in fermented products, especially dairy products, has been exhaustively explored by researchers and food industries. However, due to the growth of vegetarianism, lactose intolerance, allergy to milk proteins, and high cholesterol content of dairy products, the market prospects and the research field related to probiotic and prebiotic products are gradually changing in the last years (FARNWORTH et al., 2007, VINDEROLA, BURNS, & REINHEIMER, 2017). The replacement of milk by water-soluble soy extract in fermented soy products similar to yogurt and fermented milk has been characterized as a promising alternative in the development of new products in Brazil (ROSSI et al., 2011). Several benefits are attributed to the fermentation of the soy water-soluble extract, such as the reduction of its characteristic flavour and aroma, thus improving the sensory aspects and resulting in decreased carbohydrate levels that may be responsible for the sensation of bloating and flatulence, as well as increased free isoflavone levels (BATTISTINI et al., 2018; BEDANI et al., 2013; 2014; 2015; MONDRAGON-BERNAL et al., 2010; YEO & LIONG, 2010). In addition, when soy proteins are metabolized by probiotic microorganisms, they may produce bioactive peptides known to confer health benefits (LEE & HUR, 2017, PIHLANTO & KORNONEN, 2015, SINGH & VIJ, 2017).

Lactobacilli and bifidobacteria, alone or in combination with prebiotic ingredients, were successfully employed in several studies for the development of fermented water-soluble soy extract as soy “yoghurt” and fermented soy “milk” (FARNWORTH et al., 2007; KAUR; MISHRA; KUMAR, 2009; MASOTTI et al., 2011; PANDEY & MISHRA, 2015), soy “yoghurts” and fermented soy “milk” with soybean by-product (okara) (BEDANI; ROSSI; SAAD, 2013; BEDANI et al., 2014; 2015), fermented beverage based on vegetable soybean (BATTISTINI et al., 2018), soy-based “cheese”, soy frozen dessert, and soy ice-cream (HEENAN et al., 2004; MATIAS et al., 2014; 2016; LIU et al., 2006).

CHAMPAGNE et al. (2009) evaluated the fermentation of water-soluble soybean extract with the combination of several probiotic strains with *Streptococcus thermophilus* as the starter culture. The authors observed that the synergy of growth of these probiotics with the

starter culture did not differ from that obtained in milk, thus demonstrating that there are no losses in the fermentation process when compared to the fermentation in milk. In a recently published research, PATRIGNANI et al. (2018) concluded that strains belonging to the species *Bifidobacterium aesculapii* grew well in "soymilk", producing high amounts of exopolysaccharide, and increasing the rheological and sensorial quality of fermented "soymilk". An increased growth and viability of the strain *Lactobacillus acidophilus* LA-5 in fermented "soymilk" with different concentration of apple juice was shown by ÍÇIER et al. (2015), since the authors noted a variation in probiotic populations between 8.7 to 9.1 log CFU/g during refrigeration storage. However, MATIAS et al. (2014) observed that the same strain presented a significant decrease of up to ~2 log CFU/g in its count during the soy-based petit-suisse cheese storage. ALBUQUERQUE et al. (2017) observed that the supplementation of fermented "soymilk" with passion-fruit by-product and fructo-oligosaccharides (FOS) increased the population of *L. acidophilus* LA-5, *L. rhamnosus* LGG, and *L. reuteri* RC-14, but did not influence the population of the *L. fermentum* PCC and *Streptococcus thermophilus* strains, except for strain TA-40, which decreased in the presence of fruit by-product and FOS. In addition, the authors showed that the use of passion-fruit by-product and FOS in fermented "soymilk" contributed to the growth of probiotics and starter cultures and to increase the production of natural folate. In contrast, BATTISTINI et al. (2018) demonstrated that inulin and FOS supplementation in fermented soymilk did not increase the viable counts of *L. acidophilus* LA-5 and *B. animalis* BB-12 during storage, with population ≥ 5.6 log CFU/g and ≥ 8 log CFU/g, respectively. WU et al., (2012) reported that *Propionibacterium frudenrichii* subsp. *sheramanii* ATCC 13673 increased the survival of *Bifidobacterium adolescentis* Int57 in a fermented "soymilk". In this line, a high viability of *L. acidophilus* LA-5 and *B. animalis* BB-12, as well as a great survival of *B. animalis* BB-12 under gastrointestinal stress was reported by BEDANI, ROSSI, and SAAD (2013) in a fermented soy product during refrigerated storage. Nevertheless, in a later study BEDANI et al. (2014) showed that the presence of tropical fruit pulp (mango and guava) did not affect the *L. acidophilus* LA-5 and *B. animalis* BB-12 viabilities in a fermented soy product, but there was a significant decrease in the probiotic survival to simulated gastrointestinal stress for both probiotic strains, *L. acidophilus* LA-5 and *B. animalis* BB-12. In an earlier study, SHIMAKAWA et al. (2003) evaluated the probiotic potential of the water-soluble soybean extract fermented with *Bifidobacterium breve* YIT 4065 and noted that the populations of this microorganism reached 1.6 x 10^9 CFU/mL, remaining stable for a period of 20 days at 10 °C. The researchers suggested that the soy protein

may have exerted a protective effect, increasing the survival of *B. breve* cells when they were exposed to the action of bile.

Regardless of the controversial results previously described about probiotic survival in the soy-based food matrix, several have shown many health effects attributed to the consumption of these products like the reduction of cardiovascular disease risk (BEDANI et al., 2015; DONG et al., 2016; PADHI et al., 2016; SIMENTAL-MENDÍA et al., 2018; ROSSI et al., 1999; 2003), immunomodulatory activity (MASOTTI et al., 2011; LIN et al., 2016), decrease in the immune-reactivity to soy proteins by *L. helveticus* fermentation (MEINLSCHMIDT et al., 2016), decreased formation of putrefactive compound by the gut microbiota (NAKATA et al., 2017), reduction on the risk of breast and colorectal cancer (BEDANI et al., 2010; KINOUCHI et al., 2012; SIVIERI et al., 2008), and also, contribution for the decrease in the risk for the development of postmenopausal osteoporosis (BEDANI et al., 2006; SHIGUEMOTO et al., 2007).

In view of what was previously discussed in this chapter, the incorporation of fruit and vegetable by-products in a soy-based product (entirely of vegetal origin) could represent a promising alternative for the reduction of the disposal of food industrial by-products with high nutritional and biological properties, with the aggregation of value to them. Additionally, the combination of the functional properties of the fruit residues and the water-soluble soy extract together with probiotic microorganisms may result in a fermented multifunctional soy product with great potential for modulation and modification of the human intestinal microbiota.

3. OBJECTIVES

3.1. General Objective

- To develop a fermented soy beverage added of fruit or vegetable by-products, supplemented with probiotic strains and to evaluate their impact on the composition and metabolic activity of the human gut microbiota, employing the *in vitro* model TIM-2.

3.2. Specifically objectives

- To select the best industrial by-products (fruit and soy) and amaranth flour, based on their physical-chemical, microbiological, technological, and functional characteristics.
- To select probiotic strains to be employed in fermented soy beverages, based on their salt bile deconjugation and proteolytic activity, as well as their ability to ferment raffinose (one of the soy carbohydrate responsible for bloating and flatulence after the consumption of soy products).

- To develop a fermented soy product with the probiotic strains and the by-product previously selected and to evaluate its microbiological, technological, and sensory characteristics during 28 days of refrigeration storage (4 ºC).
- To evaluate the probiotic survival in the fermented soy product developed using an *in vitro* static model and to assess the effect of the by-product selected on the probiotic *in vitro* survival.
- To evaluate the impact of the soy fermented product on the composition and metabolic activity of the intestinal microbiota of obese individuals, compared to that of lean ones, using an in vitro model that simulate the colon conditions (TIM-2).

4. REFERENCES[1]

AAGAARD, K. et al. The placenta harbors a unique microbiome. **Science Translational Medicie**, v.6, n.237, 2014.

AGROCLUSTER RIBATEJO. **Tendências do Mercado alimentar do Brasil:** Aspectos do consumo, produto, distribuição e comunicação. Serie: Estudo de tendências de consumo em mercado internacionais e novas tecnologias: Brasil. Portugal, 2015. Available in: http://downloads.agrocluster.com/482016911506904. Acessed in: 05.12.2017.

AJILA, C.M. et al. Mango peel powder: a potential source of antioxidant and dietary fiber in macaroni preparations. **Innovative Food Science and Emerging Technologies**, v.11, p.219-224, 2010.

AJILA, C.M.; LEELAVATHI, K.; RAO, U.J.S.P.; Improvement of dietary fiber content and antioxidant properties in soft dough biscuits with the incorporation of mango peel powder. **Journal of Cereal Science**, v.48, p.319-326, 2008.

ALBUQUERQUE, M.A.C. et al. Passion fruit by-product and fructooligosaccharides stimulate the growth of folate production by starter and probiotic cultures in fermented soymilk. **International Journal of Food Microbiology**, v.261, p.35-41, 2017.

ALBUQUERQUE, M.A.C. et al. Supplementation with fruit and okara soy by-products and amaranth flour increases the folate production by starter and probiotic culture. **International Journal of Food Microbiology,** v.236, p.26-32, 2016.

ALIAKBARIAN, B. et al. Production of a novel fermented milk fortified with natural antioxidants and its analysis by NIR spectroscopy. **LWT – Food Science and Technology**, v.62, p.376-383, 2015.

AMBALAM, P. et al. Probiotic, prebiotic and colorectal cancer prevention. **Best Practice & Research Clinical Gastroenterology**, v.30, p.119-131, 2016.

AMERICAN DIETETIC ASSOCIATION. Position of the American Dietetic Association: Functional Foods. **Journal of the American Dietetic Association**, v.109, p.735-746, 2009.

ASEMI, Z. et al. Effects of synbiotic food consumption on metabolic status of diabetic patients: A double-blind randomized cross-over controlled clinical trial. **Clinical Nutrition**, v.33, p.198-203, 2014.

[1] References according ABNT rules'

BÄCKHED, F. et al. Dynamics and Stabilization of the human gut microbiomes durin the first year of life. **Cell Host & Microbe**, v.17, p.690-703, 2015.

BAGAROLLI, R.A. et al. Probiotics modulate gut microbiota and improve insulin sensitivity in DIO mice. **Journal of Nutritional Biochemistry**, v.50, p.16-25, 2017.

BANERJER, J. et al. Bioactives from fruit processing wastes: Green approaches to valuable chemicals. **Food Chemistry**, v.225, p. 10-22, 2017.

BARROS, H.R.M.; FERREIRA, T.A.P.C.; GENOVESE, M.I. Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. **Food Chemistry**, v.134, p.1892-1898, 2012.

BATTISTINI, C., et al. Development and characterization of an innovative synbiotic fermented beverage based on vegetable soybean. **Brazilian Journal of Microbiology**, v.49, n.2, p.303-309, 2018.

BEDANI, R. et al. Effect of fermented soy product on the fecal microbiota of rats fed on a beef-based animal diet. **Journal of the Science Food and Agriculture**, v.90, p.233-238, 2010.

BEDANI, R. et al. Incorporation of soybean by-product okara and inulin in a probiotic soy yoghurt: texture profile and sensory acceptance. **Journal of the Science of Food and Agriculture***,* v.94, n.1, p.119-125, 2013.

BEDANI, R. et al. Influence of daily consumption of synbiotic soy-based product supplemented with okara soy by-product on risk factors for cardiovascular diseases. **Food Research International**, v.73, p.142–148, 2015.

BEDANI, R. et al. Tropical fruit pulps decreased probiotic survival to *in vitro* gastrointestinal stress in synbiotic soy yoghurt with okara during storage. **LWT-Food Science and Technology**, v.55, p.436-443, 2014.

BEDANI, R.; et al. Efeito do consumo de um novo produto fermentado de soja enriquecido com isoflavonas e cálcio sobre o tecido ósseo de ratas maduras ovariectomizadas. **Archivos Latinoamericanos de Nutrición**, v. 56, p.146-152, 2006.

BEDANI, R.; ROSSI, E.A.; SAAD, S.M.I. Impact of inulin and okara on Lactobacillus acidophilus LA-5 and Bifidobacterium animalis Bb-12 viability in a fermented soy product and probiotic survival under in vitro simulated gastrointestinal conditions. **Food Microbiology**, v.34, p.382-389, 2013.

BEGUM, P.S. et al. Probiotics as functional foods: Potential effects on human health and its impact o neurological diseases. **International Journal of Nutrition, Pharmacology, Neurological Diseases**, v.7, p.23-33, 2017.

BEN JEDDOU, K. et al. Improvement of texture and sensory properties of cakes by addition of potato peel powder with high level of dietary biber and protein. **Food Chemistry**, v.217, p.668-677, 2017.

BENÍTEZ, V. et al. Onion (*Allium cepa* L.) by-products as source of dietary fiber: physicochemical properties and effect on serum lipid levels in high-fat fed rats. **European Food Research and Technology**, v.234, p.617-625, 2012.

BIASUCCI, G. et al. Mode of delivery affects the bacterial community in the newborn gut. Eary Human Development, v.86, p.S13-S15, 2010.

BRUCE-KELLER, A.; SALBAUM, J.M.; BERTHOUD, H.-R. Harnessing gut microbes for mental health: getting from here to there. **Biological Psychiatry**, v.83, p.214-223, 2018.

BUROLAS, A. et al. Targeting the microbiota-gut-brain axis: Prebiotic have anxiolytic and antidepressant-like effects and reverse the impact of chronic stress in mice. **Biological Psychiatry**, v.82, p.472-487, 2017.

BUTEL, M.-J. Probiotic, gut microbiota and health. **Médecine et Maladies Infectieuses**, v.44, n.1, p.1-8, 2014.

CHAMPAGNE, C.P. et al. Selection of probiotic bacteria for the fermentation of a soy beverage in combination with *Streptococcus thermophilus*. **Food Research International**, v.42, p.612-621, 2009.

CHAVES-LÓPEZ, C. et al. Control of household mycoflora in fermented sousages using phenolic fractions from olive mill wastewaters. **International Journal of Food Microbiology**, v.207, p.49-56, 2015.

CHU, D. et al. Profiling of microbiota in second trimester amniotic fluid reveals a distinctive community present in the mid trimester and predictive of the placenta microbiome at parturition. **American Journal of Obstetrics & Gynecology**, v.216, n.1, p.S18, 2017. Supplement.

COLLADO, M.C. et al. Human gut colonisation may be initiated *in utero* by distinct microbial communities in the placenta and amniotic fluid. **Scientific Reports**, v.6, p.1-13, 2016.

D'ANGENIO, V.; SALVATORE, F. The role of the gut microbiome in the healthy adult status. **Clinica Chimica Acta**, v.451, p.97-102, 2015.

DIGIULIO, D.B. et al. Microbial prevalence, diversoty and abuncance in amniotic fluid during preterm labor: a molecular and culture-based investigation. **PlosOne**, v.3, n.8, e3056, 2008.

DIGIULIO, D.B. et al. Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of mambranes. **American Journal of Reproductive Immunology**, v.64, p.38-57, 2010.

DINAN, T.G.; CRYAN, J.F. Melancholic microbes: a link between gut microbiota and depression? **Neurogstroenterology & Motility**, v.25, p.713-719, 2013.

DOGRA, S. et al. Rate of establishing the gut microbiota in infancy has consequences for the future health. **Gut Microbes**, v.6, n.5, p.321-325, 2015.

DONG, S. et al. Soy milk powder supplemented with phytotesrol ester reduced serum cholesterol level in hypercholesterolemia independently of lipoprotein E genotype: a random clinical placebo-controlled trial. **Nutrition Research**, v.36, p.879-884, 2016.

DONKOR, O.N. et al. Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. **International Dairy Journal**, v.17, p.657-665, 2007.

DUZZIONI, A.G. et al. Effect of drying kinetics on main bioactive compounds and antioxidant activity of acerola (*Malpighia emarginata* D.C.) residue. **International Journal of Food Science and Technology**, v.48, p.1041-1047, 2013.

ESHAK, N.S. Sensory evaluation and nutritional value of balady flat bread supplemented with banana peels as a natural source of dietary fiber. **Annals of Agricultural Science**, v.61, n.2, 229-235.

ESPÍRITO SANTO, A.P. et al. Fiber from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. **International Journal of Food Microbiology,** v.154, p.135-144, 2012.

FARNWORTH, E.R. et al. Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. **International Journal of Food Microbiology**, v.116, p.174–181, 2007.

FERNANDES, R. et al. Effects of inulin-type fructans, galacto-oligosaccharides, and related synbiotic on inflammatory markers in adult patients with overweight or obesity: A systematic review. **Clinical Nutrition**, v.36, p.1197-1206, 2017.

FERNÁNDEZ-GINÉS, J.M. et al. Effect of storage conditions on quality characteristics of Bologna sausages mad with citrus fiber. **Journal of Food Science**, v.68, n.2, (2003).

GALANAKIS, C.M. Emerging technologies for the production of nutraceuticals from agricultural by-products: A viewpoint of opportunities and challenges. **Food and Bioproducts Processing**, v.91, p.575-579, 2013.

GIBSON, G.R. et al. The Ineternatinal Scientific Association for Probiotic and Prebiotics (ISAPP) consensus statement on the definition ans scope of prebiotics. **Narute Reviews Gastroenterology & Hepatology**, v.14, n.8, p.491-502, 2017.

GIBSON, G.R.; ROBERFROID, M.B. **Handbook of prebiotics**. Boca Raton: CRC Press, 2008. 485p

GUBIANI, D. et al. Outlier based literature exploration for cross-domain linking of Alzheimer's disease and gut microbiota. **Expert Systems With Applications**, v.85, p.386-396, 2017.

HEENAN, C.N. et al. Survival and sensory acceptability of probiotic microorganisms in a nonfermented frozen vegetarian dessert. **LWT – Food Science and Technology**, v.37, p.461–466, 2004.

HILL, C. et al. The International Scientific Association for Probiotic and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews Gastroenterology & Hepatology**, v.11, p.506-514. 2014.

ÌÇIER, F. et al. Changes on some quality characteristics of fermented soy milk beverage with added apple juice. **LWT – Food Science and Technology**, v.63, p.57-64, 2015.

JIMÉNEZ, E. et al. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by caesarean section. **Current Microbiology**, v.51, p.270-274, 2005.

KANG, D.-W. et al. Diffences in fecal microbial metabolites and microbiota of children with autism spectrum disorders. **Anaerobe**, v.49, p.121-131, 2018.

KAUR, H.; MISHRA, H.N.; KUMAR, P. Textural properties of mango soy fortified probiotic yoghurt: optimization of inoculum level of yoghurt and probiotic culture. **International Journal Food Science & Technology**, v.44, p.415-424, 2009.

KIM, H.-N. et al. Correlation between gut microbiota and personalty in adults: A cross-sectiona study. **Brain, Behavior, and Immunity**, v.69, p.374-385, 2018.

KIMURA, I. et al. The gur microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. **Nature Communications**, v.4, 1829, 2013.

KINOUCHI, F.L. et al. A soy-based product fermented by Enterococcus faecium and Lactobacillus helveticus inhibits the development of murine breast adenocarcinoma. **Food and Chemical Toxicology,** v.50, p.4144-4148, 2012.

KOJIMA, Y. et al. Combining prebiotics and probiotics to develop novel synbiotics that suppress oral pathogens**. Journal of Oral Biosciences**, v.58, p.27-32, 2016.

KOLIDA, S.; GIBSON, G. R. Synbiotics in health and disease. **Annual Reviews of Food Science and Technology**, v.2, p.377-393, 2011.

KOWALSKA, H. et al. What's new in biopotential of fruit and vegetable by-products applied in the food processing industry? **Trends in Food Science & Technology,** v.67, p. 150-159, 2017

LEE, S.Y.; HUR, S.J. Anthypertensive peptides from animal products, marine organisms, and plants. **Food Chemistry**, v.228, p.506-517, 2017.

LI, H. et al. Correlations of fecal metabonomic and microbiomic changes induced by hight-fat diet in the pre-obesity state. **Scientific Reports,** v.6, 21618, 2016.

LI, Q.; ZHOU, J.-M. The microbiota-gut-brain axis and its potential therapeutic role in autism spectrum disorder. **Neuroscience,** v.324, p.131-139, 2016.

LIN, Q. et al. Modulation of the TNFα-induced gene expression profile fo intestinal epithelial cells by soy fermented with lactic acid bacteria. **Journal of Functional Foods,** v.23, p.400-411, 2016.

LIU, D.-M. et al. Survivability of *Lactobacillus rhamnosus* during the Preparation of Soy Cheese. **Food Technology and Biotechnology**, v. 44, p. 417–422, 2006.

LOPES, M.A.; CONTINI, E. Agricultura, Sustentabilidade e Tecnologia. **Agroanalysis**, v. 32, n. 02, p. 27-34, 2012.

MANCUSO, C.; SANTANGELO, R. Alzheimer's disease and gut microbiota modifications: the long way between preclinical studies and clinical evidence. **Pharmacological Research**, v.129, p.329-336, 2018.

MARTINELLI, L.A. et al. Agriculture in Brazil: impacts, costs, and opportunities for a sustainable future. **Current Opinion in Enviromental Sustainability**, v.2, p.431-438, 2010.

MARTINEZ, R.C.R.; BEDANI, R.; SAAD, S.M.I. Scientific evicende for healfh effects attributed to the consumption of probiotic and prebiotics: an update for current perspectives and future challenges. **British Journal of Nutrition**, v.114, p.1993-2015, 2015.

MARTINS, Z.E.; PINHO, O.; FERREIRA, I.M.P.L.V.O. Food industry by-products used as functional ingredients of bakery products. **Trends in Food Science & Tecnology**, v.67, 106-128, 2017.

MASOTTI, A.I. et al. Immunomofulatory bioactivity of soy and milk ferments on monocyte and macrophages models**. Food Research International**, v.44, p.2475-2481, 2011.

MATIAS, N.S. et al. A probiotic soy-based innovative product as an alternative to petit-susse cheese. **LWT-Food Science and Technology**, v.59, p.411-417, 2014.

MATIAS, N.S. et al. In vitro gastrointestinal resistance of *Lactobacllus acidophilus* LA-5 and *Bifidobacterium animalis* Bb-12 in soy and/or milk-base synbiotic apple ice creams. **International Journal of Food Microbiology**, v.234, p.83-93, 2016.

MEINLSCHMIDT, P. et al. Immunoreactivity, sensory and physicochemical properties of fermented soy protein isolate. **Food Chemistry**, v.205, p.229-238. 2016.

MILDNER-SZKUDLARZ, S.; BAJERSKA, J. Protective effect of grape by-product-fortified breads against cholesterol/cholic acid diet-induced hypercholesterolaemia in rats. **Journal of Science, Food and Agriculture,** v.93, p. 3271-3278, 2013.

MIRABELLA, N.; CASTELLANI, V.; SALA, S. Current options for the valorization of food manufacturing waste: a review. **Journal of Cleaner Production**, v. 65, p.28-41, 2014.

MITSUOKA, T. Development of functional foods. **Bioscience of Microbiota, Food and Health**, v.33, n.3, p.117-128, 2014.

MOLES, L. et al. Bacterial diversity in meconium of preterm neonates and evolution of their fetal microbiota during the first month of life. **PlosOne**, v.8, n.6, e66986, 2013.

MONDRAGÓN-BERNAL, O. et al. Optimization of synbiotic fermented food from hydrosoluble soy extract applying experimental design and sensory analysis techniques. **Journal of Sensory Studies**, v.25, n.3, p.371-389, 2010.

MULLER, N.T. et al. The infant microbiome development: mom matters. **Trends in Molecular Medicine**, v.21, n.2, p.109-117, 2015.

NAKATA, T. et al. Inhibitory effects of soybean oligosaccharides and water-soluble soybean fibre on formation of putrefactive compound from soy protein by gut microbiota. **International Journal of Biological Macromolecules**, v.97, p.173-180, 2017.

O'CONNOR, S. et al. Prebiotics in the management of components of the metabolic syndrome. **Maturitas**, v.104, p.11-18, 2017.

O'SHEA, N.; ARENDT, E.K.; GALLAGHER, E. Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. **Innovative Food Science and Emerging Technologies**, v.16, p.1-10, 2012.

OECD/Food and Agriculture Organization of the United Nations (2015). **Brazilian agriculture: Prospects and challenges.** OECD-FAO Agricultural Outlook 2015, 21st ed. p. 61-119. Paris: OECD Publishing. Available in: http://dx.doi.org/10.1787/agr_outlook-2015-en/. Accessed 08.03.2017

OUWEHAND, A.C. A review of dose-responses of probiotic in human studies. **Beneficial Microbes**, v.8, n.2, p.143-151, 2017.

PADHI, E. et al. Tracking isoflavones in whole soy flour, soy muffins and the plasma of hypercholesterolaemic adults. **Journal of Functional Foods**, v.24, p.420-428, 2016.

PANDEY, S.M.; MISHRA, H.N. Optimization of the prebiotic & probiotic concentration and incubation temperature for the preparation of synbiotic soy yoghut using response surface methodology**. LWT-Food Science and Technology**, v.62, p.458-467, 2015.

PARASHAR, A.; UDAYABANU, M. Gut microbiotaa: Implications in Parkinson's disease. **Parkinsonism and Related Disorders**, v.38, p.1-7, 2017.

PIHLANT, A.; KORHONEN, H. Bioactive peptides from fermented foods and health promotion. In: W. HOLZAPFEL (Ed.). **Advanced in Fermented Foods and Beverages**. Amsterdam: Woodhead Publishing Series in Food Science, Technology and Nutrition, 2015. p. 39-74.

RASMUSSEN, H.E.; HAMAKER, B.R. Prebiotics and Inflammatory Bowel Disease. **Gastroenterology Clinics of North America**, v.46, p.783-795, 2017.

REIS, S.A. et al. Review of the mechanisms of probiotic actions in the prevention of colorectal cancer. **Nutrition Research,** v.37, p. 1-19, 2017.

ROMAN, A. et al., Amniotic fluid microbiome community and inflammation in women with second trimester cervival changes. **Journal of Obstetrics & Gynecology**, v.212, n.1, p.S153-S154, 2017. Supplement.

ROMERO, R. et al. Clinical chorioamnionitis at term I: microbiology of the amniotic cavity using culrivation and molecular techniques. **Journal of Perinatal Medicine**, v.43, n.1, p.19-36, 2015.

ROSSI, E. A. et al. Produtos probióticos e prebióticos à base de soja. In: SAAD, S.M.I.; CRUZ, A.G.; FARIA, J.A.F. (Ed.). **Probióticos e prebióticos em alimentos:** Fundamentos e aplicações tecnológicas. São Paulo: Livraria Varela, 2011. cap.22, p.541-563.

ROSSI, E.A. et al. Development of a novel fermented soymilk product with potential probiotic properties. **European Food Research Technology**, v.209, p.305-307, 1999.

ROSSI, E.A. et al. Efeito de um novo produto fermentado de soja sobre os lípides séricos de homens adultos normocolesterolêmicos. **Archivos Latinoamericanos de Nutrición**, v. 53, p. 47-51, 2003.

SÁNXHEZ, B. et al. Omics for the study of probiotic microorganisms. **Food Research International**, v.54, p.1061-1071, 2013.

SAYAS-BARBERÁ, E. et al. Combined use of a probiotic culture and citrus fiber in a traditional sausage '*Longaniza de Pascua*'. **Food Control**, v.27, p.343-350, 2012.

SEKIROV, I. et al. Gut microbiota in health and disease. **Physiological Reviews**, v.90, p.859-904, 2010.

SENDRA, E. et al. Incorporation of citrus fibers in fermented milk containing probiotic bacteria. **Food Microbiology**, v.25, p.13-21, 2008.

SHIGUEMOTO, G.E. et al. Isoflavone-supplemented soy yoghurt associated with resistive physical exercise increase bone mineral density of ovariectomized rats. **Maturitas**, v. 57, p.261-270, 2007.

SHIMAKAWA, Y. et al. Evaluation of *Bifidobaterium breve* strain Yakult-fermented soymilk as a probiotic food. **International Journal of Food Microbiology,** v.81, p.131-136, 2003.

SIMENTAL-MENDÍA, L.E. et al. Effect of soy isoflavones supplementation on plasma lipoprotein (a) concentrations: A meta-analysis. **Journal of Clinical Lipidology**, v.12, n.1, p.16-24, 2018.

SINGH, B.P.; VIJ, S. Growth and bioactive peptides production potential of *Lactobacillus plantarum* strain C2 in soy milk: A LC-MS/MS based revelation for peptides biofunctionality. **LWT-Food Science and Technology,** v.86, p.293-301, 2017.

SIVIERI, K.; et al. Probiotic *Enterococcus faecium* CRL 183 inhibit chemically induced colon cancer in male Wistar rats. **European Food Research Technology**, v. 228, p.231-237, 2008.

SOHN, K.; UNDERWOOD, M.A. Prenatal and postnatal administration of prebiotic and probiotics. **Seminars in Fetal & Neonatal Medicine**, v.22, p.284-289, 2017.

SOMMER, F.; BÄCKHED, F. The gut microbiota – masters of host development and physiology. **Nature Reviews Microbiology**, v.11, p.227-238, 2013.

SU, P.; HENRIKSSON, A.; MITCHELL, H. Prebiotics anhance survival and prolong the retention period of specific probiotic inocula in an *in vivo* murine model. **Journal of Applied Microbiology**, v.103, p.2392-2400, 2007.

TAHADADI-EBRAHIM, M. et al., Effects of daily consumption of synbiotic bread on insulin metabolism and serum high-sensitivity c-reactive protein among diabetic patients: a double-blind, randomized, controlled clinical trial. **Annals of Nutrition & Metabolism**, v.65, 34-41, 2014.

TAMANG, J.P. et al. Functional properties of microorganisms in fermented foods. **Frontiers in Microbiology**, v.7, article 578, 2016.

TANAKA, M.; NAKAYAMA, J. Development of the gut microbiota in infancy and its impact on health in later life. **Allergology International**, v.66, p.515-522, 2017.

TESTRO, A.G.; VISVANATHAN, K. Toll-like receptors and their role in gastrointestinal disease. **Journal of Gastroenterology and Hepatology**, v.24, p.943-954, 2009.

TOLLIN, K.; ERZ, A.; VEJ, J. The strategic viewpoint of innovation and marketing teams on the development of novel functional foods. In: Bagchi, D.; Nair, S. eds. **Developing new functional food and nutraceutical products.** London: Academic Press,2017, pag.63-83.

TRIPATHI, M.K.; GIRI, S.K. Probiotic functional foods: Survival of probiotics furing processing and storage. **Journal of Functional Foods,** v.9, p.225-241, 2014.

TUR, J.A.; BIBILONI, M.M. Functional foods. In: CABALLERO, B.; FINGLAS, P.M.; TOLDRÁ, F. eds. **Encyclopedia of Food and Health**, Cambridge: Academic Press, 2016, pag. 157-161.

VINDEROLA, G.; BURNS, P.; REINHEIMER, J. Probiotics in Nondairy Products. In: MARIOTTI, F. (Ed.). **Vegetarian and plant-based diets in health and disease prevention**, London: Academic Press, 2017. p. 809-835.

VIUDA-MARTOS, M. et al. Effect of adding citrus waste water, thyme and oregano essential oil on the chemical, physical and sensory characteristics of a bologna sausage**. Innovative Food Science and Emerging Technologies**, v. 10, p.655-660, 2009.

VUONG, H.E.; HSIAO, E.Y. Emerging roles for the gut microbiome in autism spectrum disorder. **Biological Psychiatry**, v.81, p.411-423, 2017.

YEO, S.-K.; LIONG, M.-T. Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics. **International Journal of Food Science and Nutrition**, v.61, n.2*,* p.161-181, 2010.

ZACARÍAS, M. F. et al. Preliminary technological and potential probiotic characterization of bifidobacteria isolated from breast milk for use in dairy products**. International Dairy Journal**, v.21, n.8, p.548-555, 2011.

Chapter 2

The impact of fruit and soy by-products and amaranth on the growth of probiotic and starter microorganisms

Published as:

VIEIRA, A.D.S.; BEDANI, R.; ALBUQUERQUE, M.A.C.; BISCOLA, V.; SAAD, S.M.I. The impact of fruit and soy by-product and amaranth on the growth of probiotic and starter microorganisms. **Food Research International**, v.97, p. 356-363, 2017.
[https://doi.org/10.1016/j.foodres.2017.04.026]

ABSTRACT

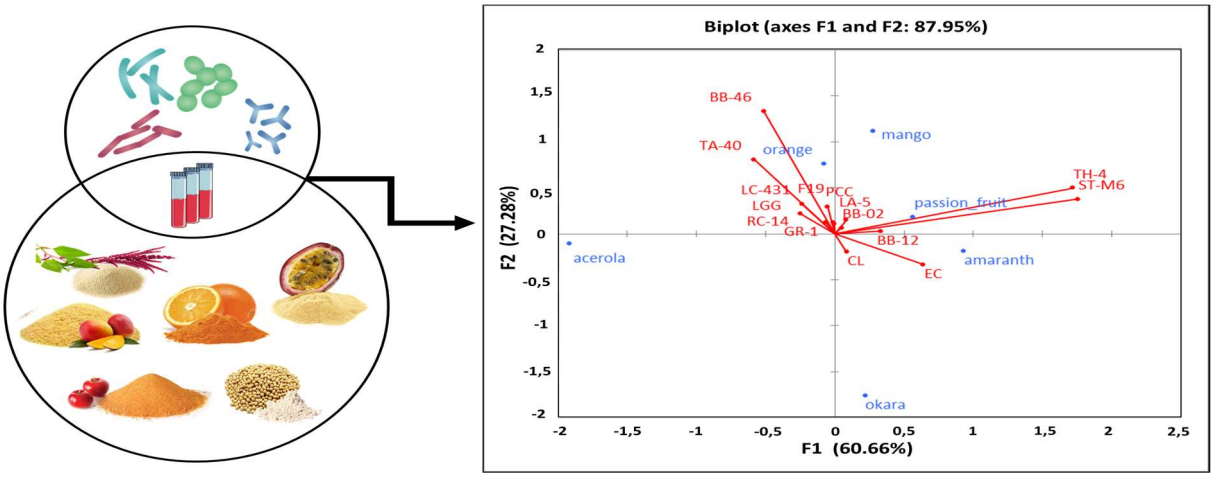
The ability of different fruit by-products, okara, and amaranth flour, to support the growth of probiotic and non-probiotic strains was evaluated. The tests were conducted with three commercial starter cultures (*Streptococcus thermophilus*), ten probiotic strains (seven *Lactobacillus* spp. and three *Bifidobacterium* spp. strains), and two harmful bacteria representative of the intestinal microbiota (*Escherichia coli* and *Clostridium perfringens*). *In vitro* fermentability assays were performed using a modified MRS broth supplemented with different fruits (acerola, orange, passion fruit, and mango), and soy (okara) by-products or amaranth flour. Orange and passion-fruit by-products were the substrates that most promoted the growth of bacterial populations, including pathogenic strains. On the other hand, the acerola by-product was the substrate that showed the highest selectivity for beneficial bacteria, since the *E. coli* and *Cl. perfringens* populations were lower in the presence of this fruit by-product. Although the passion fruit by-product, okara, and amaranth stimulated the probiotic strains, the growth of the pathogenic strains studied was higher compared to other substrates. Different growth profiles were verified for each substrate when the different strains were compared. Although pure culture models do not reflect bacterial interaction in the host, this study reinforces the fact that the ability to metabolize different substrates is strain-dependent, and acerola, mango, and orange by-products are the substrates with the greatest potential to be used as prebiotic ingredients.

Keywords: Probiotic; Fruit by-products; Okara; Amaranth; Prebiotic; Fermentability.

Highlights

- Fermentability of fruit by-products, okara, and amaranth flour was evaluated.
- Orange and passion fruit by-products promoted the highest growth of strains.
- Acerola by-product showed higher selectivity for growth of beneficial strains.
- Okara and amaranth flour stimulated the growth of *E.coli* and *Cl. perfringens*.
- Selective fermentation was strain-dependent and influenced by the substrate.

Graphic abstract



1. INTRODUCTION

For more than two decades, Brazilian agriculture has registered strong growth. Brazil has become a major exporter of agricultural products, with a surplus of USD 78.6 billion in 2013. The country is one of the largest fruit exporters and the largest exporter of processed citrus, particularly concentrated frozen orange juice. In addition to citrus fruits, the main fruits produced include bananas, apples, grapes, melons, and tropical fruits, particularly papayas, mangoes, avocados, and pineapples. These last three are the most important in terms of volume. In the grain sector, soy are expected to continue to be one of the most important agricultural products. Currently, Brazil is the second largest producer, only behind the USA, but this scenario is expected to change by 2024, with Brazil overtaking the USA (OECD/FAO, 2015).

By-products generated in the fruit and vegetable processing industries are also an important environmental problem, resulting in significant economic losses for the sector. These facts have increased the interest of the food industries in discovering and applying strategies to improve the sustainability of food processing, such as the use of these by-products for livestock feeding and fuel production (Villanueva-Suárez, Pérez-Cózar, & Redonco-Cuenca, 2013). Even though they are frequently treated as industrial waste, they might be good sources of nutrients and bioactive compounds and improve the nutritional and functional properties of food products. A good example of this is okara, a by-product of soymilk and tofu (bean curd) processing, which presents high amounts of dietary fibres, proteins, lipids, and minerals, along with unspecified monosaccharides and oligosaccharides (Jiménez-Escrig, Tenorio, Espinosa-Martos, & Rupérez, 2008; Mateos-Aparicio, Mateos-Peinado, Jiménez-Escrig, & Rupérez, 2010). In general, okara may be considered a good and cheap source of dietary fibres, since they are its major component (Lu, Liu, & Li, 2013), and could be used to increase the content of high-value compounds in different products (Bedani, Campos, Castro, Rossi, & Saad, 2014; Villanueva-Suárez et al., 2013).

The majority of by-products generated by the fruit industry are made up of peel, seeds, and skims (O'Shea, Arendt, & Gallagher, 2012). Peel constitutes around 15-20% of the fresh fruit, and some studies have demonstrated that this is also a promising source of health-promoting compounds and could be used in functional foods (Ajila, Aalami, Leelavathi, & Rao, 2010; Barros, Ferreira, & Genovese, 2012). Passion fruit peel, for example, is rich in dietary fibre (DF), especially pectin (Zeraik, Pereira, Zuin, & Yariwake, 2010). By-products from orange juice production have also been widely studied and applied as a DF source in various products, such as bakery products (Romero-Lopez, Osorio-Diaz, Bello-Perez, Tovar, & Bernardino-Nicanor, 2011), functional dairy products (Sendra, Fayos, Lario, Fernández-Lopes,

Sayas-Barberá, & Pérez-Alvarez, 2008), and meat products (Sayas-Barberá, Viuda-Martos, Fernández-López, Pérez-Alvarez, & Sendra, 2012). Acerola, as well as passion fruit and orange by-products, presents significant amounts of DF, especially pectin, polyphenols, anthocyanins, phenolic compounds, and α-glucosidase inhibitors (Assis, Lima, & Oliveira, 2001; Paz et al., 2015; Rochette et al., 2013). Mango by-products have important bioactive compounds to improve health and reduce the risk of diseases, and studies have demonstrated their use as food ingredients in many products (Ajila et al., 2010; Jahurul et al., 2015).

Dietary fibres are largely present in fruit by-products generated during processing and their potential to increase the population of beneficial bacteria, including *Lactobacillus* spp. and *Bifidobacterium* spp. has been recognized (Silva, Cazarin, Bogusz Junior, Augusto, & Maróstica Junior, 2014). Soluble fibres may form gels in the gastrointestinal tract, which contribute to their fermentability by the gut microbiota. The products generated during this fermentation may be associated with different effects on the host's health and well-being, such as increasing colon bacteria biomass and reducing colonic pH through the production of short-chain fatty acids. These compounds are important for the nutrition of enterocytes and the inhibition of pathogenic bacteria (Gibson, 2004).

Another potential substrate that could be used as a fermentable functional ingredient is amaranth (*Amaranthus* spp.) seed flour. Amaranth is a highly nutritional pseudocereal, which is an excellent source of proteins and other nutrients, including dietary fibre, vitamins, and minerals (Caselato-Sousa & Amaya-Farfán, 2012). *In vitro* and *in vivo* assays have shown that hydrolysis of amaranth proteins leads to the release of bioactive peptides with potential antithrombotic and antihypertensive activities (Fritz, Vecchi, Rinaldi, & Añón, 2011; Sabbione, Nardo, Añón, & Scilingo, 2016).

Therefore, as plant substrates may have compounds which might be promising in terms of stimulating the growth of beneficial bacteria, the present study aimed to evaluate the fermentation of different vegetable (fruit and soy) by-products, as well as amaranth flour by probiotic (lactobacilli and bifidobacteria) and starter (streptococci) strains and by harmful bacteria representative of the intestinal microbiota (*Escherichia coli* ATCC 8739 and *Clostridium perfringens* ATCC 13124).

2. MATERIAL AND METHODS

2.1. Amaranth flour and the manufacture of the industrial by-products powder

Orange, acerola, passion fruit, and mango by-products were obtained from fruit processing companies located in the state of São Paulo, Brazil (in March, July, August, and

December 2014, respectively) and stored at -18 ºC until their processing. Peel and seeds were the fruit by-products employed, except for mango, where only peel was used. All frozen fruit by-products were thawed at 4 ºC for 48 h, washed, bleached (for 12 min), and dried in an air flow oven (60 ºC/24 h). A blender (Magiclean, Arno, São Paulo, Brazil) was used to reduce the dry fruit by-products into a fine powder, and sieves were used to standardize the particle sizes (diameter below 0.42 mm). The final by-product powders were stored in polypropylene bags and kept at -18 ºC until use. Okara was supplied by UNIVERSOJA (Production and Development Unit for Soy Derivatives) located at the School of Pharmaceutical Sciences of the São Paulo State University (Araraquara, São Paulo state, Brazil) according to Bedani, Rossi & Saad, (2013). The amaranth flour (Vida Boa, Produtos Naturais, Limeira, Brazil) was obtained from a local store (São Paulo, Brazil). The fruit and okara by-products powder (≤ 0.42 mm) and amaranth flour were processed and sterilized by gamma irradiation following Albuquerque, Bedani, Vieira, LeBlanc, & Saad (2016).

2.2. Physicochemical analysis of the by-products and amaranth flour

Total solids, lipids, protein (N x 6.25), and ash were determined according to the methods described by IAL (2008). Carbohydrate content was calculated by difference to reach 100 g of total contents. Total dietary fibre, soluble fibre, and insoluble fibre quantifications were conducted at the Physicochemical Laboratory of the Food Intelligence Laboratories Inc. (São Paulo, Brazil), using the enzymatic-gravimetric method 991.43 (AOAC, 2003).

2.3. Bacterial growth in the presence of fruit or soy by-products, or amaranth flour

Thirteen food-grade strains normally used as probiotic or starter cultures by the food manufacturers, as well as two harmful bacteria representative of the intestinal microbiota, were evaluated using an *in vitro* fermentability test, according to García-Cayuela et al. (2014), Ryu, Kim, Park, Lee, & Lee (2007), and Watson et al. (2012). Briefly, fresh cultures of each strain (obtained at 37 ºC for 24 h in the cultivation conditions described in **Table 2.1**), were individually inoculated (4-5 log CFU) in formulated modified DeMan-Rogosa-Sharpe (MRS) broth with phenol red (mMRS, Ryu et al., 2007), individually supplemented with 1% (w/v) of each by-product or amaranth flour. The enumeration of the bacterial populations was performed before (0 h) and after 24 h, and 48 h of incubation at 37 ºC, using the appropriate agar media for each microorganism (**Table 2.1**).

Table 2.1. Microorganisms evaluated in the present study and conditions employed for the cultivation of each strain.

Strains	Code	Broth	Agar	Incubation
Streptococcus thermophilus TH-4[a]	TH-4	HJ[1]	M17[2]	37 °C Aerobic
St. thermophilus ST-M6[a]	ST-M6			
St. thermophilus TA-40[b]	TA-40			
Lactobacillus acidophilus LA-5[c]	LA-5	MRS[3]	MRS with maltose[4]	37 °C Aerobic
L. fermentum PCC[c]	PCC	MRS[3]	MRS[5]	
L. reuteri RC-14[c]	RC-14			
L. paracasei subsp. *paracasei L. casei* 431[c]	431	MRS[3]	acidified MRS (pH 5.4)[6]	37 °C Anaerobic[7]
L. paracasei subsp. *paracasei* F19[c]	F19			
L. rhamnosus GR-1[c]	GR-1			37 °C Aerobic
L. rhamnosus LGG[c]	LGG			
Bifidobacterium animalis subsp. *lactis* BB-12[c]	BB-12	MRS cysteine (0.05%)[8]	LP-MRS[9]	37 °C Anaerobic[7]
B. longum BB-46[c]	BB-46			
B. longum subsp. *infantis* BB-02[c]	BB-02			
Escherichia coli ATCC 8739	EC	BHI[10]	EMB[10]	37 °C Aerobic
Clostridium perfringens ATCC 13124	CL	RCM[10]	RCA[11]	37 °C Anaerobic[7]

[a] Starter culture (Chr. Hansen, Hørsholm, Denmark); [b] Starter culture (DuPont, Dangé, France); [c] Probiotic cultures (Chr. Hansen); 1. Hogg-Jago Glucose (HJ) (Blomqvist, Steinmoen, & Håvarstein, 2006); 2. M17 agar (Oxoid, Basignstoke, UK) (Richter & Vedamuthu, 2001); 3. De Man-Rogosa and Sharpe broth; 4. MRS modified agar supplemented with maltose (IDF, 1995); 5. MRS agar (Oxoid); 6. Acidified MRS agar pH 5.4 (Buriti, Cardarelli, & Saad, 2007); 7. Anaerobes System AnaeroGen™ (Oxoid); 8. MRS Broth (Oxoid) supplemented with L-cysteine (0.05 g/L, Sigma-Aldrich, St. Louis, USA); 9. LP-MRS agar (Vinderola, Prosello, Ghiberto, & Reinheimer, 2000); 10. RCM broth, BHI broth, EMB agar (Oxoid), 11. RCM broth added to 13.5 g/L bacterial agar (Oxoid).

2.4. Statistical analysis

The Shapiro-Wilk test was used to verify the data normality, and the Bartlett test was used to check assumptions of homoscedasticity. In order to evaluate the global effect of vegetable substrates on bacterial growth, in addition to chemical composition, data were submitted to Variance Analysis (ANOVA), and the Tukey test was used for means comparison. Data that did not show normality and homoscedasticity were submitted to Kruskal-Wallis and

to means comparison by the Fisher LSD test. The statistical package Statistica 13.0 (StatSoft, Tulsa, OK, USA) was employed, and the results were presented as means ± standard deviation (SD). Principal Components Analysis was performed to evaluate the relationships between each bacterial strain and the vegetable substrates after 24 h and 48 h of incubation. Covariance was the matrix type used in the mapping. For this purpose, the XLSTAT 2017 software was employed.

3. RESULTS

3.1. Chemical composition of the by-products and of amaranth flour

The chemical composition of each by-product and amaranth flour is presented in **Table 2.2.** A significant variation in total solids (TS) between all the by-products evaluated and amaranth flour ($P<0.05$) after drying was observed. This variation in TS is probably due to the smaller size of the acerola and passion fruit fragments, compared to those of orange and mango, which resulted in a larger contact area, leading to higher mass transfer during the drying process. Regarding protein content, okara and amaranth powders presented the highest values (35.25 g/100 g and 13.13 g/100 g, respectively), in comparison with all the fruit by-products studied ($P<0.05$).

Table 2.2. Chemical composition of fruit by-products, okara, and amaranth powders.

Parameters (g/100 g)	Acerola	Mango	Orange	Passion fruit	Okara	Amaranth
Total solids	98.35(0.09)^{B}	89.87(0.19)^{F}	96.27(0.05)^{C}	99.55(0.07)^{A}	95.52(0.11)^{D}	93.93(0.08)^{E}
Protein	8.23(0.21)^{C}	5.14(0.15)^{E}	6.55(0.08)^{D}	8.59(0.71)^{C}	35.25(0.25)^{A}	13.13(0.25)^{B}
Ash	2.57(0.04)^{B}	2.57(0.27)^{B}	3.38(0.17)^{A}	2.34(0.16)^{B}	3.09(0.04)^{A}	2.26(0.09)^{B}
Fat	2.33(0.07)^{D}	1.93(0.44)^{D}	2.38(0.18)^{D}	11.19(0.64)^{B}	19.84(0.08)^{A}	6.99(0.28)^{C}
Total carbohydrates (including TDF[a])	85.21(0.38)^{A}	80.22(0.32)^{C}	83.95(0.24)^{B}	77.43(0.73)^{D}	37.35(0.12)^{F}	71.57(0.53)^{E}

$^{A-F}$ Different superscript capital letters in a row denote significant differences ($P<0.05$) between different by-products and/or amaranth. [a]Total dietary fibres. Values are expressed as means of repetitions (standard deviation)

The acerola by-product presented the highest total dietary fibre (TDF) content (48.46 g/100 g), of which only 0.59 g/100 g corresponded to the soluble fraction (SF), and 47.87 g/100 g to the insoluble fraction (IF). The passion fruit by-product also presented a low amount of SF (0.12 g/100 g) and a high IF content (42.11 g/100 g), reaching a TDF content of 42.23 g/100 g. Neither okara nor amaranth powder presented SF in their composition, and the TDF content

completely corresponded to the IF content of, respectively, 35.32 g/100 g and 11.78 g/100 g. The mango and orange by-products presented, respectively, 41.53 g/100 g and 42.92 g/100 g (TDF); 11.37 g/100 g and 7.46 g/100 g (SF); and 30.16 g/100 g and 35.46 g/100 g (IF). The total carbohydrate content of okara (37.35 g/100 g) completely corresponded to the IF fraction, while acerola, mango, orange, and passion fruit by-products, as well as amaranth, respectively presented 85.21 g/100 g, 80.22 g/100 g, 83.95 g/100 g, 77.43 g/100 g, and 71.57 g/100 g of total carbohydrates, indicating that, besides SF and IF these by-products also presented simple carbohydrates.

3.2. *In vitro* fermentability of fruit/soy by-products and amaranth flour

In general, higher bacterial populations ($P<0.05$) were observed in the mMRS medium with the orange and passion fruit by-products (**Figure 2.1**). The bacterial populations in mMRS medium supplemented with mango by-product and amaranth were not statistically different from the orange and passion fruit by-products, and nor were they significantly different ($P>0.05$) from the acerola by-product and okara, which presented the lowest growth compared to the media supplemented with orange and passion fruit by-products. As expected, each vegetable substrate presented a different selective effect on the growth of bacterial strains studied. Supplementation of the culture broth with the fruit by-products, okara, and amaranth flour resulted in increased populations for most of the strains studied (**Figure 2.2 A-F)**. Higher populations after incubation in the presence of all the fruit by-products evaluated, okara, and amaranth flour were observed for all *Lactobacillus* strains. However, *L. acidophilus* LA-5, followed by *L. rhamnosus* LGG, were the strains that presented the lowest growth in the presence of the vegetable substrates studied. When comparing 24 h and 48 h of incubation, there was a significant reduction ($P<0.05$) in populations of the following strains: *L. acidophilus* LA-5, in the presence of acerola, passion fruit by-products, and okara; *L. rhamnosus* LGG, in the presence of the acerola by-product and okara, as well as *L. rhamnosus* GR-1, in the presence of the acerola by-product. *Bifidobacterium animalis* BB-12 and *B. longum* BB-02 showed a significant increase ($P<0.05$) in mMRS broth supplemented with all substrates tested. On the other hand, *B. longum* BB-46 grew significantly ($P<0.05$) only in fruit by-products, except for the passion fruit by-product in the first 24 h of assay. In fact, *B. longum* BB-46 populations in the presence of this by-product were lower than the non-supplemented mMRS broth (negative control) (6.83 log CFU/mL and 6.86 log CFU/mL for 24 h and 48 h, respectively, data not shown). However, after 48 h, there was a significant reduction in BB-46 populations in the presence of acerola, mango, and orange by-products, reaching values equal

to or lower than those observed for the negative control. The opposite behaviour was verified for BB-46 in presence of the passion fruit by-product for the same incubation time (48 h), since this strain had higher populations than those observed in the first 24 h. This growth was significantly higher when compared to the negative control. Among the *St. thermophilus* strains employed in this study, TA-40 grew only in the presence of the fruit by-products, but after 48 h there was a reduction in their populations in the presence of the mango by-product. However, this population reduction was significantly lower or equal to the reduction observed for the negative control (6.18 log CFU/mL and 6.28 log CFU/mL for 24 h and 48 h, respectively, data not shown). *Streptococcus thermophilus* ST-M6 showed an increase in populations in the media supplemented with all vegetable substrates, except the acerola by-product, as well as okara after 48 h of incubation. However, only for the passion fruit supplemented medium these populations were significantly higher than those observed in the negative control (7.21 log CFU/mL and 7.14 log CFU/mL for 24 h and 48 h, respectively, data not shown). Additionally, TH-4 did not show any significant increase ($P<0.05$) in the presence of by-products and/or amaranth flour. Low populations of *St. thermophilus* strains and *B. longum* BB-46 in media supplemented with the acerola by-product and okara could explain the lower global bacteria populations for these substrates supplementation.

Concerning the growth of other important microorganisms from the human intestinal tract, *Cl. perfringens* ATCC 13124 did not grow significantly in the presence of acerola, mango or orange by-products, after 24 or 48 h of incubation ($P<0.05$), when compared to the non-supplemented mMRS broth negative control (7.62 log CFU/mL and 7.68 log CFU/mL for 24 h and 48 h, respectively, data not shown). However, the broths supplemented with the passion fruit by-product, okara, and amaranth showed high *Cl. perfringens* populations after 24 h of incubation (8.31 log CFU/mL, 8.68 log CFU/mL and 8.43 log CFU/mL, respectively). The *E. coli* strain was able to grow in media supplemented with the fruit by-products, okara or amaranth flour. When comparing 24 h and 48 h, *E. coli* ATCC 8786 had the lowest growth rates in the first 24 h for all substrates studied, except for the supplemented okara medium, where populations between 24 h and 48 h of incubation did not show a significant difference ($P>0.05$). Additionally, for the same incubation time, the broth supplemented with the acerola by-product showed the lowest *E. coli* populations compared to the other substrates employed in this study ($P<0.05$).

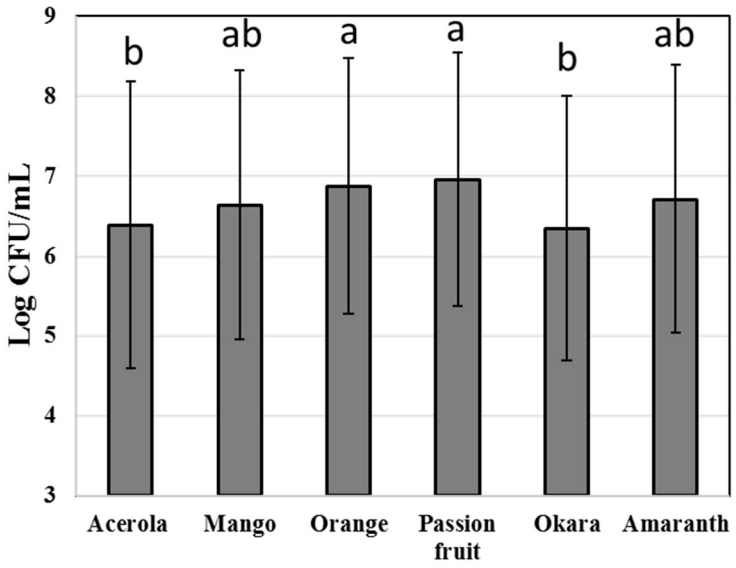


Figure 2.1. Global bacterial populations on phenol red MRS broth supplemented with 1% w/v of the acerola, mango, orange, passion fruit by-products, okara, and amaranth flour. [a,b] Different lowercase letters indicate significant differences (*P<0.05*) between different vegetables substrates independently of time of incubation and/or strain.

To evaluate which of the by-products/amaranth presented important differences regarding the growth of the strains studied, we carried out a principal component analysis (PCA) on a dataset of 15 strains using 6 vegetable substrates. **Figure 2.3** illustrates the growth of the strains after 24 h (3A) and 48 h (3B) of incubation at 37 ºC. The first principal component (PC, F1), for 24 h of incubation, explained 60.66% of the total variance between the by-products/amaranth, and the second component (F2) explained 27.28%. Therefore, the first two PC explained 87.95% of the variability between by-products/amaranth in relation to bacterial growth (**Figure 2.3 A**). For the growth after 48 h of incubation, the first PC (F1) explained 52.32% of the variability between the by-products/amaranth and the second component 36.56%. Therefore, the first two PC explained 88.87% of the variability between by-products/amaranth regarding bacterial growth (**Figure 2.3 B**).

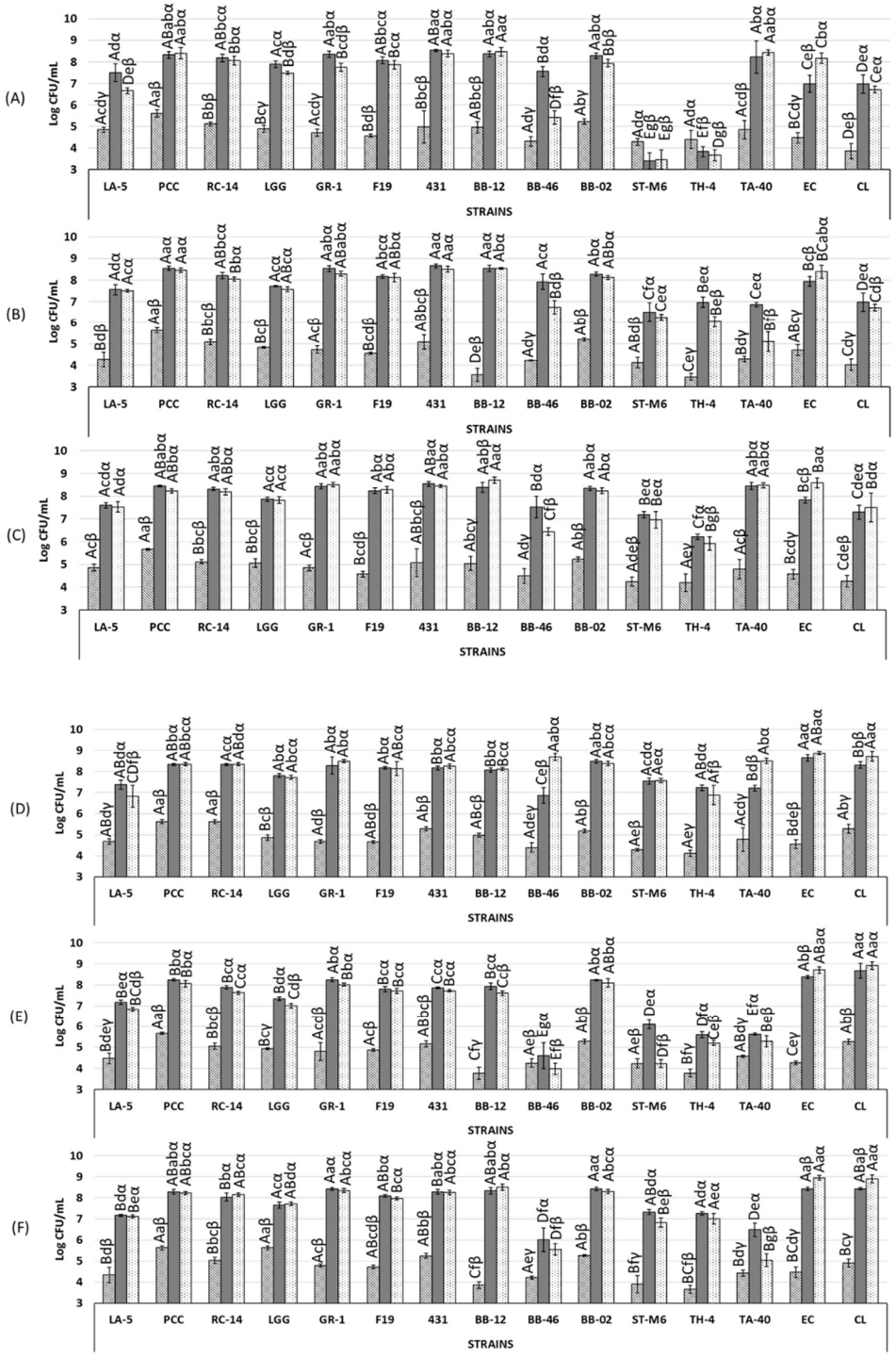


Figure 2.2. Populations of probiotic, starter, *E. coli* (EC), and *Cl. perfringens* (CL) strains* on phenol red MRS broth supplemented with 1% w/v of the (A) acerola, (B) mango, (C) orange, (D) passion fruit, (E) okara by-products, and of (F) amaranth, before (0 h, ▨) and after 24 h (■) and 48 h (⬚) of incubation at 37 °C. *See **Table 2.1** for description of strains. $^{A-G}$ Different capital letters indicate significant differences ($P<0.05$) between the different by-products and/or amaranth for a same strain in the same incubation period. $^{a-g}$ Different lowercase letters indicate significant differences ($P<0.05$) between different strains for the same by-product and/or amaranth in the same incubation period. $^{\alpha-\gamma}$ Distinct Greek letters indicate significant differences ($P<0.05$) between different incubation periods for the same strain and the same by-product and/or amaranth.

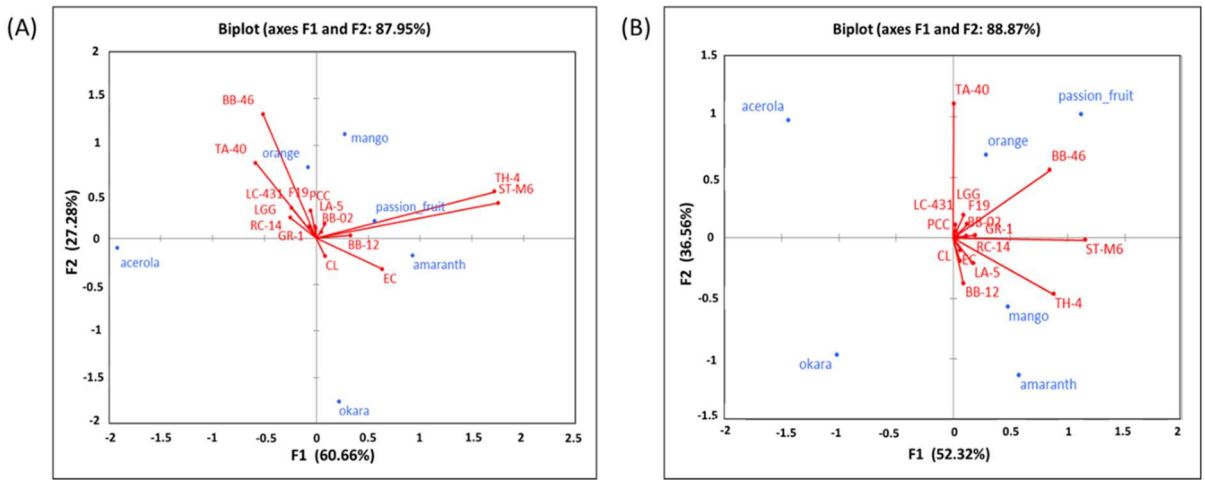


Figure 2.3. Principal Component graph of bacterial* growth in the presence of different vegetable substrates after 24 h (A) and 48 h (B) of incubation at 37 ºC. *See **Table 2.1** for description of strains.

After 24 hours of incubation, *St. thermophilus* TH-4 and ST-M6 grew better in amaranth flour, passion fruit and mango by-products, especially when compared to the acerola by-product. On the other hand, the growth of *B. longum* BB-46 and *St. thermophilus* TA-40 was higher in orange, mango, and acerola by-products, mainly when compared to the okara by-product. After 48 h of incubation, the growth of the strains BB-46, ST-M6, and TH-4 was higher in the presence of passion fruit, orange, and mango by-products and amaranth (on the right of the map, **Figure 2.3 B**). Therefore, these substrates were more favourable for the BB-46, ST-M6, and TH-4strains, compared to the acerola and okara by-products (on the left of map, **Figure 2.3 B**). BB-46 showed the highest growth in the presence of the passion fruit by-product and TH-4 in the presence of amaranth flour, whereas lower growth rates in the presence of acerola was observed for TH-4 and BB-46 in the presence of okara. However, *St. thermophilus* TA-40 showed the highest growth in the presence of acerola, orange, and passion fruit by-products, when compared to okara and mango by-products and amaranth.

4. DISCUSSION

The fermentation ability of fifteen bacteria, including three starter cultures, ten probiotic cultures, and two harmful bacteria representative of intestinal microbiota after 24 h and 48 h in modified MRS supplemented with six vegetable substrates (acerola, mango, orange, passion fruit by-products, okara, and amaranth flour) were evaluated. Several studies have demonstrated that the processing of fruit and other vegetables generates a large quantity of by-products. These by-products are great sources of bioactive compounds, including dietary fibres, antioxidants, phenolic compounds, and minerals, often containing higher concentrations of

bioactive compounds than the final products (Ajila et al., 2010; Ayala-Zavala et al., 2011; Barros et al., 2012; Duzzioni, Lenton, Silva, & Barrozo, 2013; O'Shea et al., 2012). Additionally, amaranth is classified as a pseudocereal and belongs to the group of the 'superfoods', presenting a nutritional composition that confers multiple benefits on the host. The potential prebiotic effect of dietary fibres present in vegetable by-products and pseudocereals have been widely explored (Gullón, Gullón, Tavaria, & Yáñez, 2016; Villanueva-Suárez, Pérez-Cózar, Mateos-Aparicio, & Redondo-Cuenca, 2016).

High concentrations of dietary fibre were reported by Espírito Santo et al. (2012) and Martinez et al. (2012) for passion fruit and mango by-products. Macagnan et al. (2015) have also observed the concentration of 54.82% of TDF, of which 29.65% were of IF and 25.17% of SF. In the present study, the lowest levels of TDF, SF, and IF were observed for orange by-product. Extrinsic factors such as climate, fertilisation, and soil types, as well as intrinsic factors like cultivation conditions, vegetable maturity, and even different parts of the same plant, are known to influence the chemical composition of different by-products (Assis et al., 2001; Barros et al., 2012).

A considerable amount of carbohydrates not digested by the human digestive enzymes reaches the colonic regions, where they act as the main source of energy for the colonic microbiota, altering the gut microbiota composition and the gene expression encoding proteins and enzymes of metabolic pathways involved in the use of fibres and the production of potentially beneficial metabolites such as short-chain fatty acids (SCFA) (Benítez-Páez, 2016; Rios-Covian, Gueimonde, Duncan, Flint, & Reyes-Gavilan, 2015; Scott, Gratz, Sheridan, Flint, & Duncan, 2013). Studies have shown that several fruit by-products and others vegetable substrates are sources of dietary fibre and other compounds which present a potential prebiotic effect, stimulating the gut microbiota modulation and increasing the proportion of beneficial bacteria such as *Bifidobacterium* spp., *Lactobacillus* spp., and *Faecalibacterium prautsnitzii* (Bialonska et al., 2010; Cantu-Jungles, Cipriani, Iacomini, Hamaker, & Cordeiro, 2017; Gullón et al., 2016; Maccaferri et al., 2012). Moreover, the increased survival of probiotic microorganisms in functional foods and the production of bioactive molecules like folate, lactate, and acetate in dairy or vegetable matrixes have also been described for vegetable substrates (Albuquerque et al., 2016; Bedani et al., 2013; Bialonska et al., 2010; Cantu-Jungles et al., 2017; Gullón et al., 2016). Gullón et al. (2016) evaluated the fermentability of amaranth through an *in vitro* model that used human faeces and a concentration of 1% (m/v) of amaranth in the fermentation medium. The results showed that this pseudocereal had a potential prebiotic

effect by increasing the population of selected groups present in the intestinal microbiota, such as *Bifidobacterium* spp. and *Lactobacillus/Enterococcus*.

Similarly to what has been observed in the present study regarding the acerola by-product, Bialonska et al. (2010) observed that the pomegranate by-product significantly enhanced the growth of bifidobacteria and lactobacilli, whereas populations of the pathogenic microorganism groups *Clostridium histolyticum* and *Eubacterium rectale-Clostridium coccoides* did not increase. On the other hand, the results of the present study demonstrate that this stimulus cannot be extrapolated to all members of a genus or cluster, since the *B. longum* BB-46 strain did not show the same growth as the other *Bifidobacterium* strains or even the *Lactobacillus* strains studied in the presence of amaranth. Villanueva-Suárez et al. (2013) evaluated the production of short-chain fatty acids (SCFA) during the fermentation of okara treated enzymatically with Ultraflo L® (to obtain a product with a higher proportion of soluble fibres) by a pure culture of *Bifidobacterium bifidum* DSM 20239. The authors reported a decrease in pH and substrate consumption, which demonstrated the bifidogenic capacity of okara.

In general, in the present study, supplementation of the medium with passion fruit by-products, okara or amaranth lead to the highest populations of the *Cl. perfringens* and *E. coli* strains. A possible explanation for this fact is that the high concentration of proteins present in these powders (**Table 2.2**) may have contributed to the growth of these microorganisms, since none of the other strains studied showed any proteolytic activity (data not shown). The acerola by-product was shown to have the same concentration of protein as passion fruit, but the fermentation of these proteins may have been hampered by the higher concentration of phenolic compounds of the acerola by-product (data not shown). In fact, Scott et al. (2013) reported that, among the members of the gut microbiota, *Clostridium* spp. and *E. coli* are bacteria involved in the deamination of amino acids and protein fermentation. *Streptococcus thermophilus* strains TA-40, ST-M6, and TH-4, as well as *Bifidobacterium* BB-46, presented the highest differences among the by-products. TH-4 and ST-M6 presented similar results at 24 and 48 hours when comparing different by-products. On the other hand, BB-46 stood out at 48 h for passion fruit and at 24 h for mango, orange, and acerola, and TA-40 did not stand out at 24 h for the passion fruit by-product, in which the strain showed growth promotion after 48 h of incubation.

Therefore, the results of the present study reinforce the fact that the fermentation and the potential modulation of individual strains possibly present in the gut microbiota may be influenced by different growth conditions and the availability of nutrients. This was also demonstrated by Albuquerque et al. (2016), when investigating the effect of the same vegetable

substrates on folate production by the same probiotic and starters employed herein. The authors observed that culture media supplemented with the orange by-product increased the folate production in all strains evaluated, with increments in folate contents varying between 8 ng/mL and 748 ng/mL, due to the presence of, respectively, *Lactobacillus paracasei* F19 and *Lactobacillus reuteri* RC-14. The authors reported high folate production by *Bifidobacterium longum* BB-02 and BB-46 in the presence of different by-products and amaranth flour, with BB-02 reaching increments of 1223 ng/mL in the folate content in the presence of amaranth flour. Following this line, Espírito Santo et al. (2012) studied probiotic yoghurts with fibres from fruit by-products and observed that banana and apple fibres increased the viability of *B. animalis* Bl04, HN019, and B94 and of *L. acidophilus* L10, whereas passion fruit fibre did not show any effect. Supplementation with banana, apple, and passion fruit fibres did not show any influence on *St. thermophilus* populations. In contrast, we observed an increased growth for all strains studied in the presence of the passion fruit by-product. An increased growth in the presence of passion fruit and mango by-products, as well as amaranth flour, was observed for *St. thermophilus* TH-4 and ST-M6, and in the presence of passion fruit, mango, and orange by-products for TA-40. Since the yogurt is a more complex matrix, the simple carbohydrates present, in the case of Espírito Santo et al. (2012), were probably sufficient for maintaining the *Streptococcus thermophilus* viability, thus not requiring the use of fruit by-products, different from the culture media used in the present study, where the main source of carbohydrates was one of the by-products tested, or amaranth.

Among the vegetable substrates evaluated, orange and passion fruit by-products presented the highest stimulus for the growth of all strains. Although the acerola by-product promoted lower growth in these strains, this substrate showed a high selectivity for beneficial strains over undesirable bacteria, in the case of the present study *E. coli* and *Cl. perfringens*. Besides, media supplemented with passion fruit by-product, okara, and amaranth flour stimulated the growth of these strains, which might be a drawback in their application as potential prebiotic ingredients. In this context, orange, mango, and acerola by-products could be used as promising candidates for novel sources of prebiotic ingredients, helping to minimize the problem of suitable food processing, thereby reducing the accumulation of agricultural waste. Additionally, this study suggests that selective fermentation is strain-dependent and influenced by the type of substrate available for bacterial growth. Further studies, involving the effects of fruit by-products, okara, and amaranth on the composition and metabolism of bacteria from the gut microbiota are required. Nonetheless, the present study elucidates which of the

vegetable substrates studied are more promising for the application in future studies with gut microbiota.

Acknowledgements

The study is part of the "Bioactive components from by-products of food processing used in a synbiotic approach for improving human health and well-being (BioSyn)" project, within the frame of the international thematic project "Strategic Research Collaboration in Food Science in the State of São Paulo, Brazil and Denmark – 2013" FAPESP/DCSR.The authors wish to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Projects 2013/50506-8 and 2013/19346-4),Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support and fellowships. The authors also wish to thank Christian Hansen and DuPont Brasil Ltda for kindly providing the microorganisms, Dr. Susy Sabato, Elizabeth Somessari, and Carlos Gaia from IPEN (Instituto de Pesquisas Energéticas e Nucleares) for the irradiation of the vegetable samples used in this study, Prof. Bernadette Franco, head of the Food Microbiology Laboratory at the School of Pharmaceutical Sciences of University of São Paulo, for providing some of the equipment necessary to carry out this study, and Kátia Silva for her technical assistance.

5. REFERENCES

Ajila, C.M., Aalami, M., Leelavathi, K., & Rao, U.J.S.P. (2010). Mango peel powder: a potential source of antioxidant and dietary fiber in macaroni preparations. *Innovative Food Science & Emerging Technologies*, *11*, 219-224.

Albuquerque, M.A.C. de, Bedani, R., Vieira, A.D.S., LeBlanc, J. G., & Saad, S.M.I. (2016). Supplementation with fruit and okara soy by-products and amaranth flour increases the folate production by starter and probiotic culture. *International Journal of Food Microbiology*, *236*, 26-32.

AOAC (2003). *Official methods of analysis of AOAC International*. (17th ed. 2003). Gaithersburg, MD, USA: AOAC International.

Assis, S.A., Lima, D.C., & Faria Oliveira, O.M.M. (2001). Activity of pectinmethylesterase, pectin content and vitamin C in acerola fruit at various stages of fruit development. *Food Chemistry*, *74*, 133-137.

Ayala-Zavala, J.F., Vega-Vega, V., Rosas-Domínguez, C., Palafox-Carlos, H., Villa-Rodriguez, J.A., Siddiqui, Md.W., et al. (2011). Agro-industrial potential of exotic fruit byproducts as a source of food additives. *Food Research International,44*, 1866-1874.

Barros, H.R.M., Ferreira, T.A.P.C., & Genovese, M.I. (2012). Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. *Food Chemistry*, *134*, 1892-1898.

Bedani, R., Campos, M.M.S, Castro, I.A., Rossi, E.A., & Saad, S.M.I. (2014). Incorporation of soy by-product okara and inulin in a probiotic soy yoghurt: texture profile and sensory acceptance. *Journal of the Science of Food and Agriculture*, *94*, 119-125.

Bedani, R., Rossi, E.A., & Saad, S.M.I. (2013). Impact of inulin and okara on *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under *in vitro* simulated gastrointestinal conditions. *Food Microbiology*, *34*, 382-389.

Benítez-Páez, A., Del Pulgar, E.M.G., Kjølbæk, L., Brahe, L.K., Astrup, A., Larsen, L. et al. (2016). Impact of dietary fiber and fat on gut microbiota re-modeling and metabolic health. *Trends in Food Science & Technology, 57,* 201-212.

Bialonska, D., Ramnani, P., Kasimsetty, S.G., Muntha, K.R., Gibson, G.R., & Ferreira, D. (2010). The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. *International Journal of Food Microbiology, 140*, 175-182.

Blomqvist, T., Steinmoen, H., & Håvarstein, L.S. (2006). Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*. *Applied and Environmental Microbiology*, *72*, 6751-6756.

Buriti, F.C.A., Cardarelli, H.R., & Saad, S.M.I. (2007). Biopreservation by *Lactobacillus paracasei* in coculture with *Streptococcus thermophilus* in potentially probiotic and synbiotic fresh cream cheeses. *Journal of Food Protection*, *70*, 228-235.

Cantu-Jungles, T.M., Cipriani, T.R., Iacomini, M., Hamaker, B.R., & Cordeiro, L.M.C. (2017). A pectic polysaccharide from peach palm fruits (*Bactris gasipaes*) and its fermentation profile by the human gut microbiota *in vitro. Bioactive Carbohydrates and Dietary Fibre*, *9*, 1-6.

Caselato-Sousa, V.M., & Amaya-Farfán, J. (2012). State of knowledge on amaranth grain: a comprehensive review. *Journal of Food Science*, *77*, R93-R104.

Duzzioni, A.G., Lenton, V.M., Silva, D.I.S., & Barrozo, M.A.S. (2013). Effect of drying kinetics on main bioactive compounds and antioxidant activity of acerola (*Malpirghia emarginata* D. C.) residue. *International Journal of Food Science and Technology*, *48*, 1041-1047.

Espírito Santo, A.P.do, Cartolano, N.S., Silva, T.F., Soares, F.A.S.M., Gioielli, L.A., Perego, P., et al. (2012). Fiber from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. *International Journal of Food Microbiology*, *154*, 135-144.

Fritz, M., Vecchi, B., Rinaldi, G., & Añón, M.C. (2011). Amaranth seed protein hydrolysates have *in vivo* and *in vitro* antihypertensive activity. *Food Chemistry*, *126*, 878-884.

García-Cayuela, T., Díez-Municio, M., Herrero, M., Martínez-Cuestam M. C., Peláez, C., Requena, T., et al. (2014). Selective fermentation of potential prebiotic lactose-derived oligosaccharides by probiotic bacteria. *International Dairy Journal*, *38*, 11-15.

Gibson, G.R. (2004). Fibre and effects on probiotics (the prebiotic concept). *Clinical Nutrition Supplements*, *1*, 25-31.

Gullón, B., Gullón, P., Tavaria, F. K., & Yáñez, R. (2016). Assessment of the prebiotic effect of quinoa and amaranth in the human intestinal ecosystem. *Food & Function*, *14;7(9),* 3782-3788.

IAL (2008). Analytical standards of the Instituto Adolfo Lutz: physicochemical methods for food analysis. http://www.ial.sp.gov.br/resources/editorinplace/ial/2016_3_19/analisedealimentosial_2008.pdf/Accessed 15.02.2015.

IDF (1995). Fermented and non-fermented milk products. Detection and enumeration of *Lactobacillus acidophilus*. FIL-IDF Standard 306 Culture media Brussels: International Dairy Federation.

Jahurul, M.H.A., Zaidul, I.S.M., Ghafoor, K., Al-Juhaimi, F.Y., Nyam, K.-L., Norulaini N.A. N. et al. (2015). Mango (*Mangifera indica* L.) by-products and their valuable components: a review. *Food Chemistry*, *183*, 173-180.

Jiménez-Escrig, A., Tenorio, M.D., Espinosa-Martos, I., & Rupérez, P. (2008). Health-promoting effects of dietary fibre concentrate from the soy by-product okara in rats. *Journal of Agricultural and Food Chemistry*, *56*, 7495-7501.

Lu, F., Liu, Y., & Li, B. (2013). Okara dietary fibre and hypolycemic effect of okara foods. *Bioactive Carbohydrates and Dietary Fibre*, *2*, 126-132.

Macagnan, F.T., Santos, L.R. dos, Roberto, B.S., Moura, F.A. de Bizzani, M., & Silva, L.P. da. (2015). Biological properties of apple pomace, Orange bagasse and passion fruit peel as alternative sourse of dietary fibre. *Bioactive Carbohydrates and Dietary Fibre*, *6*, 1-6.

Maccaferri, S., Klinder, A., Cacciatore, S., Chitarrari, R., Honda, H., Luchinat, C. et al. (2012). *In vitro* fermentation of potential prebiotic flour from natural sources: Impact on the human colonic microbiota and metabolome. *Molecular Nutrition & Food Research, 56*, 1342-1352.

Martinez, R., Torres, P., Meneses, M.A., Figueroa, J.G., Pérez-Álvarez, J.A., & Viuda-Martos, M. (2012). Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dieaty fibre concentrate. *Food Chemistry*, *135*, 1520-1526.

Mateos-Aparicio, I., Mateos-Peinado, C., Jiménez-Escrig, A., & Rupérez, P. (2010). Multifunctional antioxidant activity of polysaccharide fractions from the soy by-product okara. *Carbohydrate polymers*, *82*, 245-250.

O'Shea, N., Arendt, E.K., & Gallagher, E. (2012). Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. *Innovative Food Science and Emerging Technologies*, *16*, 1-10.

OECD/Food and Agriculture Organization of the United Nations (2015). Brazilian agriculture: Prospects and challenges. OECD-FAO Agricultural Outlook 2015, 21st ed. p. 61-119. Paris: OECD Publishing. http://dx.doi.org/10.1787/agr_outlook-2015-en/Accessed 08.03.2017

Paz, M., Gúllon, P., Barroso, M.F., Carvalho, A.P., Domingues, V.F., Gomes, A.M., Becker, H., et al. (2015). Brazilian fruit pulps as functional foods and additives: evaluation of bioactive compounds. *Food Chemistry*, *172*, 462-468.

Richter, R.L. & Vedamuthu, E.R. (2001). Compendium of the methods for the microbiological examination of foods, 4th ed. p. 483–495. Washington, DC: APHA.

Rios-Covian, D., Gueimonde, M., Duncan, S.H., Flint, H.J., & Reyes-Gavilan, C.G. de los. (2015). Enhanced butyrate formation by cross-feeding between *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis*. *FEMS Microbiology Letters, 362(21),* fnv176

Rochette, N.F.G., Mota, E.F., Nunes-Pinheiro, D. C. S., Bezerra, C. F., Oliveira, M. L. M., Silva, A. C. M., et al. (2013). Effect of the pre-treatment with acerola (*Malpighia emarginata* DC.) juice on ethanol-induced oxidative stress in mice – hepatoprotective potential of acerola juice. *Free Radicals and Antioxidants*, *3*, S16-S21.

Romero-Lopez, M.R., Osorio-Diaz, P., Bello-Perez, L.A., Tovar, J., & Bernardino-Nicanor, A. (2011). Fibre concentrate from orange (*Citrus sinensis* L.) bagase: Characterization and application as bakery product ingredient. *International Journal of Molecular Sciences*, *12*, 2174-2186.

Ryu, S.I., Kim, B.G., Park, M.S., Lee, Y.B., & Lee, S.B. (2007). Evaluation of enhanced hygroscopicity, bifidogenicity, and anticariogenicity of enzymatically synthesized β-galactosyl-trehalose oligosaccharides. *Journal of Agricultural and Food Chemistry*, *55*, 4184-4188.

Sabbione, A.C., Nardo, A.E., Añón, M.C., & Scilingo, A. (2016). Amaranth peptides with antithrombotic activity released by simulated gastrointestinal digestion. *Journal of Functional Foods*, *20*, 204-214.

Sayas-Barberá, E., Viuda-Martos, M., Fernández-López, F., Pérez-Alvarez, J.A., & Sendra, E. (2012). Combined use of a probiotic culture and citrus fibre in a traditional sausage 'Longaniza de Pascua'. *Food Control*, *27*, 343-350.

Scott, K.P., Gratz, S.W., Sheridan, P.O., Flint, H.J., & Duncan, S.H. (2013). The influence of diet on the gut microbiota. *Pharmacological Research*, *69*, 52-60.

Sendra, E., Fayos, P., Lario, Y., Fernández-Lopes, J., Sayas-Barberá, E., & Pérez-Alvarez, J. A. (2008). Incorporation of citrus fibres in fermented milk containing probiotic bacteria. *Food Microbiology*, *25*, 13-21.

Silva, J.K., Cazarin C.B.B., Bogusz Junior, S., Augusto, F., & Maróstica Junior, M.R. (2014). Passion fruit (*Passiflora edulis*) peel increases colonic production of short-chain fatty acids in Wistar rats. *LWT - Food Science and Technology*, *59*, 1252-1257.

Villanueva-Suárez, M.J., Pérez-Cózar, M.L., & Redonco-Cuenca, A. (2013). Sequential extraction of polysaccharides from enzymatically hydrolyzed okara byproduct: physicochemical properties and *in vitro* fermentability. *Food Chemistry*, *141*, 1114-1119.

Villanueva-Suárez, M.J., Pérez-Cózar, M.L., Mateos-Aparicio, I., & Redondo-Cuenca, A. (2016). Potential fat-lowering and prebiotic effect of enzymatically treated okara in high-cholesterol fed Wistar rats. *International Journal of Food Sciences and Nutrition*, 67, 828-833.

Vinderola, C.G., Prosello, W., Ghiberto, D., & Reinheimer, J.A. (2000). Viability of probiotic (*Bifidobacterium, Lactobacillusacidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in Argentinian fresco cheese. *Journal of Dairy Science*, *83*, 1905–1911.

Watson, D., Motherway, M.O.C., Schoterman, M.H.C., Neerven, R.J.J.V., Nauta, A., & Sinderen, D.V. (2012). Selective carbohydrate utilization by lactobacilli and bifidobacteria. *Journal of Applied Microbiology*, *114*, 1132-1146.

Zeraik, M.L, Pereira, C.A.M, Zuin, V.G, Yariwake, J.H. (2010). Passion fruit: a functional food? *Brazilian Journal of Pharmacognosy*, *20*, 459-471.

Chapter 3

Impact of acerola by-product and probiotic strains on technological and sensory features of a fermented soy beverage.

Manuscript to be submitted for publication as:

VIEIRA, A.D.S.; BISCOLA, V.; BEDANI, R.; ALBUQUERQUE, M.A.C.; SAAD, S.M.I. *Impact of acerola by-product and probiotic strains on technological and sensory features of a fermented soy beverage*

ABSTRACT

Thirteen food-grade strains (seven *Lactobacillus*, three *Bifidobacterium*, and three *Streptococcus thermophilus* strains) were screened for capacity to hydrolyse cow's milk and soy proteins; ferment raffinose; and deconjugate bile salts (BSD) for application in a fermented soy beverage (FSB) produced with acerola by-product (ABP). None of the tested strains presented any proteolytic activity. *Streptococcus thermophilus* TH-4 presented the best growth rates in the presence of raffinose. Amongst lactobacilli and bifidobacteria strains, the best bile salt deconjugation (BSD) activities were observed for *L. acidophilus* LA-5 and *B. longum* BB-46, respectively. LA-5 and BB-46 were also tested for their effect on technological features and sensory acceptability of a fermented soy beverage (FSB) produced with acerola by-product (ABP). BB-46 and ABP negatively affected FSB acceptability. ABP also led to significant differences in FSB instrumental texture ($P<0.05$). Besides, the presence of ABP influenced in the product's moisture ($P<0.05$), leading to an increase in firmness and decreasing its sensory acceptability. These facts did not influence the beverage's purchase intention since FSB4 (the beverage containing ABP) obtained the best scores for this criteria.

Keywords: acerola by-product, bile salt hydrolyses, fermented soy products, probiotic, sensory acceptability

Highlights:

- *L. acidophilus* LA-5 and *B. longum* BB-46 were able to deconjugated bile salts;
- *S. thermophilus* TH-4 presented high growth rates in the presence of raffinose;
- None of the tested strains presented proteolytic activity;
- Fermented soy beverage presented high contents of linoleic acid;
- Acerola by-product and *B. longum* reduced the sensory acceptability of soy beverages.

1. INTRODUCTION

The first processed foods consumed by humans were fermented products. Biotransformation that takes place during fermentation leads to the modification of the raw material into a new product. Besides, these processes are also able to increase the nutritional, sensorial, and functional proprieties of the food products (Marco et al., 2017). During the last decades, the use of beneficial bacteria as probiotic strains in fermented products, especially dairy products, has been exhaustively explored by researchers and food industries. However, some factors such as the increase of vegetarianism, lactose intolerance, cow's milk allergy and the high amounts of cholesterol from dairy products, are increasing the interest towards vegetal products and leading to a gradual change in the market and the research of probiotics (Farnworth et al., 2007, Vinderola, Burns, & Reinheimer, 2017).

Studies have been reporting that vegetable-based products as fruit juices, vegetable "milk" (from soy and cereals), and tea are great alternatives to substitute milk as vehicles of probiotics (de Lacey, Pérez-Santín, López-Caballero, & Montero, 2014, Farnworth et al., 2007, Rivera-Espiniza & Gallardo-Navarro, 2010). Proteins, polyunsaturated fatty acids, dietary fibre, and isoflavones are amongst the main functional compounds found in soy-based foods which are known to promote health. Moreover, these compounds present an important role in the reduction of the risk of cardiovascular diseases and hypercholesterolemia (Bedani, Vieira, Rossi, & Saad, 2014, Bedani et al., 2015, Fukuda, Kobayashi, & Honda, 2017). However, soybean protein presents high amounts of enzyme inhibitors, such as proteinase and trypsin inhibitors, which can reduce its digestibility. Fermentation processes may improve the digestibility of soy proteins since the proteolytic enzymes produced by the microbial populations can hydrolyse these proteins (Sanjukta & Rai, 2016). In addition, the fermentative process can generate secondary metabolites of great technological and functional interest, such as proteolytic enzymes and bioactive peptides. Some authors have reported the antihypertensive effect of fermented soy products, the production of peptides with antioxidant activity and the reduction of the allergenicity of milk proteins (Lee & Hur, 2017, Pihlanto & Kornonen, 2015). The presence of bioactive peptides with antioxidant and angiotensin converting enzyme-inhibitory activity in soy "milk" fermented by *Lacobacillus plantarum* C2 was reported by Singh & Vij, (2017). The authors observed high *L. plantarum* in soy "milk" growth rates, followed by the generation of some bioactive peptides. Additionally, the fermentation of water-soluble soy extract by probiotic microorganisms may improve its palatability, increase its functional proprieties and reduce the levels of non-digestible oligosaccharides like raffinose and stachyose, responsible for flatulence, abdominal cramps and bloating feeling (Battistini, et

al., 2018, Bedani, Campos, Castro, Rossi, & Saad, 2013, Bedani et al., 2014, 2015, Singh & Vij, 2017, Yeo & Liong, 2010). Combined with the interesting functional properties of soy components, the use of probiotic bacteria that present one or more mechanisms of reduction of serum cholesterol represents a strategy in the prevention and treatment of hypercholesterolemia. Among these mechanisms, the production of bile salt hydrolases (BSH) is one of the most exploited and both *in vitro* and *in vivo* studies are used for this purpose. BSH is responsible for the de-conjugation of bile salt. The production of these enzymes by commensal bacteria plays a chief role on their colonization and survival in the mammalian intestine (Chand et al., 2017) and is considered as one of the main criteria in the selection of new potential probiotics bacteria (Pavlović, Stankon, & Mikor, 2012, Shehata, El Sohaimy, El-Shan, & Youssef, 2016). The consumption of products containing BSH-producing LAB can lead to the deconjugation of free bile salts, which are easily excreted in faecal material since they are less soluble (Sridevi, Vishwe & Prabhune, 2009). In an *in vivo* study, Joyce et al. (2014), reported a significant influence of BSH-producing bacteria in the regulation of cholesterol levels and weight gain of the mice.

The production of food waste covers the whole life cycle of a food, from its agricultural production, through the processing industry, the retail market, and even the final consumer. In developed countries, industrial production generates around 39% of all food waste in the production chain, consequent to the processing of foods (Galanakis, 2013). Agricultural by-products were considered for many years as undervalued substrates, generating major problems with the need for their treatment and disposal in the environment, which involves the use the several forms of energy, water, and other factors. It is estimated that every year millions of tons of residues, with great potential for utilization, are generated from the processing of fruits and vegetables (Galanakis, 2013, Kowalska, Czajkowska, Cichowska, & Lenart, 2017). Vegetable by-products represent an abundant source of bioactive compounds, such as dietary fibre, phenolic compounds, antioxidants, polyunsaturated fatty acids, vitamins, and minerals. Thus, these by-products could represent a great option to develop new ingredients to be employed in the innovative food production (Kowalska et al., 2017). Acerola (*Malpighia emarginata* D.C.), belongs to the family *Malpighaceae* and is a fruit native to West India and tropical South America. When fully mature, it presents contents ranging from 1000 to 2000 mg 100 g-1 of vitamin C, significant amounts of pectin, dietary fibre, carotenoids, anthocyanin, and phenolic compounds (Mohammed, 2011; Paz et al., 2015, Rochette et al, 2013). Studies have reported that acerola by-product presents high concentrations of bioactive compounds with favourable characteristics to the consumer's health and well-being. Among these bioactive compounds,

dietary fibres, vitamin C, and polyphenols contents stand out (Duzzeioni, Lenton, Silva, & Barroso, 2013, da Silva, Cazarin, Batista, & Maróstica Jr., 2014). Vieira, Bedani, Albuquerque, Biscola & Saad (2017) observed that acerola by-product represents a great option as a new potentially the prebiotic ingredient. The authors reported a selective growth of beneficial bacteria in presence of acerola by-product and an inhibition of harmful bacteria representative of the intestinal microbiota (*Escherichia coli* and *Clostridium perfringens*). In view of what was described above, the aim of this study was to evaluate the ability of 13 food-grade strains to ferment raffinose, to hydrolyse milk and soy proteins and to deconjugate bile salts. We selected ten probiotic strains (seven belonging to the lactobacilli genus and three belonging to the bifidobacteria genus) and three starter cultures (belonging to the *Streptococcus thermophilus* species). The selected lactobacilli and bifidobacteria strains were employed, in co-culture with the selected *S. thermophilus* strain, in the production of fermented soy beverages supplemented with acerola by-product. The effect of probiotic bacteria and acerola by-product in the technological and sensorial characteristics of these beverages was also evaluated.

2. MATERIAL AND METHODS

2.1. Microorganisms and culture conditions

The food-grade *Lactobacillus* spp. and *Bifidobacterium* spp., as well as *Streptococcus thermophilus* (starter culture) strains used were supplied by Chr. Hansen located at Hørsholm, Denmark, except for the *S. thermophilus* TA-40 strain, which was supplied by DuPont (São Paulo, Brazil). The cultures were activated at 37 ºC for 24 h in the specific broth for each strain (**Table 3.1**).

2.2. Determination of bile salt deconjugation ability

The evaluation of bile salt deconjugation ability of the probiotic and starter strains was performed according to Ugarte, Guglielmotti, Giraffa, Reinheimer, and Hynes (2006). Fresh cultures of each strain were pour-plated on specific culture medium (**Table 3.1**), individually supplemented with 0.5% (w/v) of the sodium salts of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC), and glycodeoxycholic acid (GDC) (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation (48 h at 37 ºC) in aerobic conditions for all *Streptococcus thermophilus* strains and in anaerobic conditions for the *Bifidobacterium* and the *Lactobacillus* strains (AnaeroGenTM, Oxoid, Basignstoke, UK). The formation of opaque or "cotton-flakes-like" halos around of colonies was verified, indicating a positive activity for bile

salt deconjugation. The trials were performed using five independent inocula and two plates for each bile salt, in a total of 10 plates for each strain and bile salt.

Table 3.1 Probiotic and starter strains, and their cultivation conditions.

Species	Strain code	Culture broth	Culture agar	Temperature of incubation	Incubation conditions
***Lactobacillus* (*L.*) strains**					
L. acidophilus	LA-5	MRS[1]	MRS-maltose[4]	37 ºC	Aerobic
L. fermentum	PCC	MRS	MRS[5]	37 ºC	Aerobic
L. reuteri	RC-14	MRS	MRS	37 ºC	Aerobic
L. rhamnosus	GR-1	MRS	MRS pH set 5.4[6]	37 ºC	Aerobic
L. rhamnosus	LGG	MRS	MRS pH set 5.4	37 ºC	Aerobic
L. paracasei subsp. *paracasei*	F-19	MRS	MRS pH set 5.4	37 ºC	Anaerobic[9]
L. paracasei subsp. *paracasei* *L. casei*	LC-431	MRS	MRS pH set 5.4	37 ºC	Anaerobic
***Bifidobacterium* (*B.*) strains**					
B. animalis subsp. *lactis*	BB-12	MRS cysteine[2] (0.05%, w/v)	LP-MRS[7]	37 ºC	Anaerobic
B. longum subsp. *infantis*	BB-02	MRS cysteine (0.05%, w/v)	LP-MRS	37 ºC	Anaerobic
B. longum	BB-46	MRS cysteine (0.05%, w/v)	LP-MRS	37 ºC	Anaerobic
Streptococcus (S.) thermophilus					
S. thermophilus	TH-4	HJ glucose[3]	M17[8]	37 ºC	Aerobic
S. thermophilus	ST-M6	HJ glucose	M17	37 ºC	Aerobic
S. thermophilus	TA-40	HJ glucose	M17	37 ºC	Aerobic

[1]DeManRogosa-Share broth (MRS, Oxoid, Basignstoke, UK); [2]MRS broth (Oxoid) supplemented with HCl cysteine (0.05%, w/v, Sigma-Aldrich, St. Louis, USA) [according to Vinderola, Prosello, Ghiberto, & Reinheimer (2000)]; [3]Hogg-Jago glucose broth [Blomqvist, Steinmoen, & Håvarstein, (2006)]; [4]MRS supplemented with maltose in substitution of glucose [according to IDF, (1995)]; [5]MRS agar (Oxoid); [6]MRS agar (Oxoid) pH set 5.4 with acetic acid 3 M [according to Butiri, Cardarelli, & Saad, (2007)]; [7]MRS agar (Oxoid) supplemented with 3 g/L of sodium propionate (Sigma-Aldrich) and 2 g/L of litium chlorate (Merch) [according to Vinderola et al., (2000)]; [8]M17 agar (Oxoid) [according to Richter & Vedamuthu, (2001)]; [9]Anaerobic system AnaeroGemTM (Oxoid).

2.3. Determination of the proteolytic activity of probiotic and starter strains

The proteolytic activity of probiotic and starter cultures (**Table 3.1**) was evaluated as described by El-Ghaish et al. (2010), using UHT skim milk (Molico, Nestle, Caçapava, SP, Brazil) and a solution (2% w/v) of defatted soy extract (Provesol IF, Olvebra, RS, Brazil). Briefly, the strains were reactivated (overnight at 37 ºC) twice in their specific broths, cells were collected and washed from the media by centrifugation (10,000 *xg* at 5 min, 4 ºC, Sigma 6-16K, Germany) and resuspended in the same volume of sterile saline solution (0.85% NaCl,

w/v). These cell suspensions were inoculated (5%, v/v) in skim milk and soy extract solution and the mixtures were incubated at 37 ºC for 24 h. After incubation, the mixtures were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to observe the occurrence of proteolysis. SDS-PAGE was performed according to Laemmli (1970) using a vertical slab electrophoresis cell (BIORAD Mini PROTEAM 3 System, Hercules, CA, USA). The occurrence of proteolysis was evidenced by the reduction in the intensity of the colour of the bands representing milk and soy protein fractions. **2.4. Evaluation of bacterial growth using different carbohydrates**

The ability of each strain to ferment glucose, fructose, sucrose, raffinose (Sigma-Aldrich, St. Louis, USA) and lactose (Oxoid, Basingstoke, UK)], and prebiotic fibres [inulin HP, inulin GR, and fructooligosaccharide –FOS P95 (Beneo, Orafti, Oreye, Belgium)] was performed as described by García-Cayuela et al. (2014), Ryu, Kim, Park, Lee, and Lee (2007), and Watson et al. (2013). Fresh cultures of each strain (obtained as described in **Table 3.1**), were individually inoculated (4-5 log CFU) in a formulated modified DeMan-Rogosa-Sharpe broth with phenol red (mMRS, Ryu et al., 2007), individually supplemented with 0.5% (w/v) of each carbohydrate. Non-supplemented mMRS broth was also inoculated and used as negative control. The growth of *Lactobacillus* spp. and *Streptococcus* spp. strains were monitored in sterile 96-well plates (200 μL of culture per well), using a spectrophotometer reader and incubator (Multiskan FC model 357, Thermo Scientific, China), in the following conditions: optical density reading at 620 nm (OD620nm) every 60 min, for 24 h in aerobic conditions. For *Bifidobacterium* spp. strains, incubation was carried out in anaerobic conditions (AnaeroGenTM Anaerobic System, Oxoid) and the absorbance was read (620 nm) in a spectrophotometer (Ultrospec®2000, Pharmacia Biotec, Brazil), after 0 h and 24 h, at 37ºC. The absorbance results for all strains after 0 h and 24 h were converted into log CFU/mL, using growth curves obtained for each microorganism.

2.5. Fermented soy beverage production with the application of the selected probiotic and starter cultures

2.5.1. Fresh bacterial cultures and acerola by-product origin

The acerola by-product (*Malpighia emarginata* D.C.) was supplied by a fruit processing company located in São Paulo (Brazil) and further processed to obtain the by-product (fine powder, <0.42 mm) as described by Vieira et al. (2017). The selection of the bacterial cultures to be used in the fermentation of the soy beverages was based on their abilities to ferment raffinose and deconjugate bile salts. According to these criteria, *Lactobacillus acidophilus* LA-5, *Bifidobacterium longum* BB-46, and *Streptococcus thermophilus* TH-4 were

selected. The strains were activated with two successive transfers from stock cultures to the following media: MRS broth (Oxoid) for LA-5; MRS broth (Oxoid) containing L-cysteine hydrochloride hydrate (0.05%, w/v, Sigma-Aldrich, Germany) for BB-46; and Hogg-Jago glucose broth (HJ) for TH-4. The inoculated media were incubated for 24 h at 37 ºC in aerobic conditions, except for BB-46, incubated in anaerobic jars with an anaerobic generator system (AnaeroGen®, Oxoid). After the second incubation, the inoculum was washed twice in sterile saline solution (NaCl, 0.85%, w/v), cells were collected by centrifugation at 8,600 gx for 10 min and stored in an ice bath until the production of the fermented soy beverage.

2.5.2. Experimental design and production of the fermented soy beverages.

A 2^3 factorial randomized design was employed for the production of the fermented soy beverages (FSB). Eight pilot-scale production trials were performed, in triplicates, in order to evaluate the addition of: *Lactobacillus acidophilus* LA-5 (Chr. Hansen), as Factor 1 (X1); *Bifidobacterium longum* BB-46 (Chr. Hansen), as Factor 2 (X2); and the acerola by-product (ABP), as Factor 3 (X3). The trials were evaluated considering two levels (presence or absence) during the production of FSB, according to **Table 3.2**. All FSB were fermented with the starter culture *Streptococcus thermophilus* TH-4.

Table 3.2. Description of the fermented soy beverage formulations studied, employing a 2^3 factorial randomized design.

Fermented soy formulations	Variables tested		
	***Lactobacillus acidophilus* LA-5**	***Bifidobacterium longum* BB-46**	**Acerola by-product**
	X1	***X2***	***X3***
FSB1	-	-	-
FSB2	+	-	-
FSB3	-	+	-
FSB4	-	-	+
FSB5	+	+	-
FSB6	+	-	+
FSB7	-	+	+
FSB8	+	+	+

+ = presence - = absence

2.5.3. Production of fermented soy beverages

Each of the eight pilot-scale FSB formulations was produced in batches of 2.5 L, in triplicates (three randomized different batches of the same formulation, using new soluble soy extract and inoculum). For the mixture and pasteurization of the soy base for the fermentation, a Mixer Thermomix® (Vorwerk International Strecker & Co, Switzerland) was used. The soluble soy extract (Mais Vita Pura Soja, culinary use, Yoki®, Pouso Alegre, MG, Brazil) was heated in constant agitation, up to 50 ºC, and added of sucrose (50 g/L, Coopersucar-União, Limeira, SP, Brazil) and dextrose monohydrated ST (10 g/L, Roquette, France). Heating continued until 80 ºC when carrageen gum (1 g/L, ETM3, Agargel Ind. e Com, Ltda, São Paulo, SP, Brazil) and soy extract powder (Yoki®) was added and mixed again until complete dissolution. When the mixture achieved 90 ºC, it was pasteurized (5 min at 90 ºC). For formulations FSB4, FSB6, FSB7, and FSB8 (**Table 3.2**), the ABP powder was added just before the pasteurization step. After, all soy bases were cooled in an ice bath up to 37 ºC, inoculated with the cells prepared as described in section 2.4.1, and incubated at 37 ºC in a B.O.D. incubator (TE-391, Tecnal®, São Paulo, Brazil), until achieving pH 5.5. Afterward, the fermented base was cooled to 4 ºC and kept at this temperature for approximately 16 h, when concentrated acerola juice (100 g/kg of fermented base) was added and mixed. The FSB was packaged in plastic containers and sealed with metallic covers with varnish in a sealer (Delgo Nr, 1968, Delgo Metalurgica, Cotia, Brazil). The products were stored at 4 ºC for 28 days.

2.6. Physical-chemical and microbiological assays

Moisture, protein (Nx6.25), and ash were determined according to the methods described by the IAL (2008). The lipid fraction was determined according to Bligh & Dyer (1959). Carbohydrates content was calculated by the difference to reach 100 g of total contents. Titratable acidity was performed by titration with an alkaline solution (0.11 N, NaOH), and expressed as % of lactic acid (m/v), according to AOAC method 16.023 (AOAC, 1984). After separation and quantification of the lipid content, the fatty acids were transformed in methyl esters, according to Hartman and Lago (1973), and the fatty acids profile was performed in a Varian GC gas chromatographer, model 430 GC, equipped with an automatic injector.

For the assessment of the microbiological quality and safety of the FSB used in sensorial test, determinations of *Bacillus cereus, Salmonella* spp*., Staphylococcus* DNAse positive, molds and yeasts, as well as thermotolerant microorganisms and total coliforms, were performed by the Food Microbiology Laboratory of the School of Pharmaceutical Sciences of University of São Paulo (São Paulo, Brazil), using the methods described by American Public Health Association (2001).

2.7. Instrumental texture profile (TPA) analysis and sensory evaluation

TPA was determined in quintuplicate samples of each batch (five different pots of the same batch), weekly up to 28 days of storage (4 ºC) with a TA-XT Plus Texture Analyser (Stable Micro Systems, Hasleme, UK), as described by Bedani et al. (2013).

Sensory analyses of the FSB were performed on 7 and 21 days of storage. Each fermented soy beverage was evaluated through the acceptability test, using the hybrid hedonic scale (0 = extremely disliked, 5 = neither liked nor disliked, 10 = extremely liked) (Buriti, Castro, & Saad, 2010). On each sampling day, 50 consumers (volunteers - untrained panelists) of the University of São Paulo, including teachers, students, and staff, were recruited, based on their interest and soy products consuming habits. During a session, each volunteer analysed three samples delivered monadically, containing approximately 20 g of FSB, and codified with three random digits. A total of 800 samples of FSB were evaluated by 284 volunteers (66.20% female and 33.80% male) aged from 18 to 58 years. Several volunteers participated in more than one session. The volunteers were instructed to report the sensorial attributes related to flavour, aroma, appearance, texture, and overall aspects, as well as purchase intention. The sensory analysis was approved by the Ethical Committee of the School of Pharmaceutical Science of the University of São Paulo (CAAE: 50569215.6.0000.0067).

2.8. Statistical analysis

To verify the normality and homoscedasticity of data, respectively, Shapiro-Wilk and Bartlett tests were used. Analysis of variance (ANOVA) was used for data to which normality and homoscedasticity were observed, and the Tukey's test was used for means comparison, in a 95% of confidence level. Data that did not show normality and homoscedasticity, a non-parametric ANOVA test of Kruskal-Wallis was used, combined with a Fisher LSD test for means comparison. The statistical package employed was Statistica 13.0 (StatSoft, Tulsa, OK, USA), the data were expressed as means ± standard deviation (SD). The Pearson correlation analysis was performed, and the Spearman correlation matrix was used for the comparing each sensory parameter (overall acceptance, appearance, aroma, flavour, and texture) with purchase intention. Principal Components Analysis was performed to evaluate the relations between the eight pilot-scale-marking formulations of the 2^3 factorial design (*L. acidophilus* LA-5, *B. longum* BB-46 and acerola by-product) and the sensory attributes in general (scores of 7 days plus 21 days of storage) and the effect of the storage period (7 and 21 days) independently.

Covariance was the matrix type used in the mapping. For this purpose, the XLSTAT 2017 software was employed.

3. RESULTS AND DISCUSSION

3.1.Screening of probiotic and starter cultures

3.1.1. Bile salt deconjugation ability

Ten probiotics and three starter strains (**Table 3.1**) were evaluated for their ability to deconjugate the following bile salts: taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC), and glycodeoxycholic acid (GDC), all in their sodium-salt forms and at a concentration of 0.5% (w/v) as shown in **Table 3.3**. All *Lactobacillus* and *S. thermophilus* strains had their growth inhibited in the presence of GDC. On the other hand, *Bifidobacterium* strains were able to grow in the presence of all tested bile salts. We observed that the ability to produce bile salt hydrolases is a strain-specific trait that cannot be extrapolated to genus or species. While *B. longum* BB-46 was able to deconjugate all tested bile salts, the same results were not observed for *B. longum* subsp. *infantis* BB-02 and *B. animalis* subsp. *lactis* BB-12. Similarly, Liong and Shah (2005b) observed the higher production of bile salt hydrolases for different *Bifidobacterium* species. Other authors have also reported that bile salts deconjugation is a strain-dependent characteristic (Pereira, McCartney, & Gibson, 2003, Tanaka, Doesburg, Iwasaki, & Mierau, 1999). Among the tested *Lactobacillus* strains, the ability to produce bile salt hydrolyses was more specific than it was for *Bifidobacterium* strains since both *L. acidophilus* LA-5 and *L. reuteri* RC-14 presented the ability to deconjugate bile salt (**Table 3.3**). These results are in agreement with those published by Liong and Shah, (2005a), who observed that *L. acidophilus* strains showed higher deconjugation ability than *L. casei* strains. In contrast with the results found in the present study for strains of the group *L. casei* (*L. rhamnosus* LGG and GR-1; *L. paracasei* F-19 and LC-431), several studies reported that strains belonging the species *L. casei, L rhamnosus,* and *L. paracasei* presented higher abilities of bile salt deconjugation, among other mechanisms for cholesterol reduction (Brashears, Gilliland, & Buck, 1998, Santos et al., 2015, Liong & Shah, 2005a, Miremadi, Ayyash, Sherkat, & Stojanovska, 2014).

Table 3.3. Growth and deconjugation of bile salts (0.5%, w/v) by probiotic and starter strains.

Strains[1]	Bile salt			
	GC	GDC	TC	TDC
LA-5	*g*+	*ng*	*g*+	*g*+
PCC	*g*	*ng*	*g*	*g*
RC-14	*g*+	*ng*	*g*+	*g*
LGG	*g*	*ng*	*g*	*g*
GR-1	*g*	*ng*	*g*	*g*
F19	*g*	*ng*	*g*	*g*
LC-431	*g*	*ng*	*g*	*g*
BB-12	*g*+	*g*+	*g*+	*g*
BB-46	*g*+	*g*+	*g*+	*g*+
BB-02	*g*	*g*+	*g*	*g*+
TH-4	*g*	*ng*	*g*	*g*
ST-M6	*g*	*ng*	*g*	*g*
TA-40	*g*	*ng*	*g*	*g*

Glycholic acid (GC); glycodeoxycholic acid (GDC); taurocholic acid (TC); taurodeoxycholic acid (TDC). *ng* no growth; *g* growth; *g*+ growth and bile salt deconjugation; [1] See **Table 3.1** for the description of strains.

3.1.2. Proteolytic activity

In the present study, ten probiotic strains and three *Streptococcus thermophilus* strains (see **Table 3.1**) were evaluated for their proteolytic activity against cow milk and soy proteins (**Figure 3.1**).

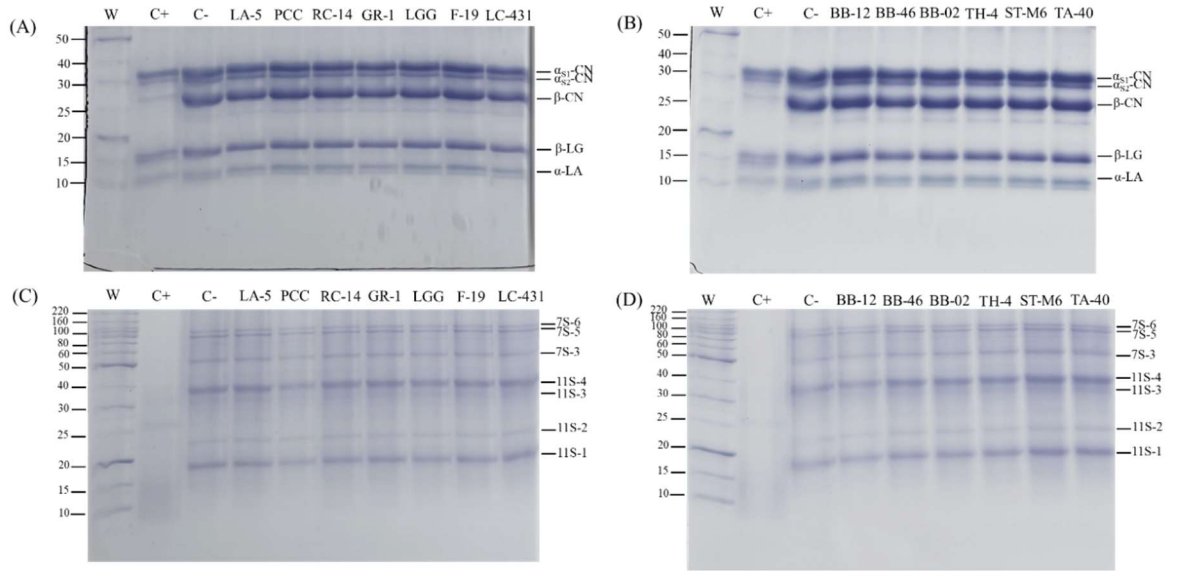


Figure 3.1. Skim milk UHT protein profiles (A, B), and soy extract proteins profiles (C, D), obtained by SDS-PAGE electrophoresis, after inoculation with different probiotic and starter strains. W = molecular weight (kDa); C+ = Positive control (*Enterococcus faecalis* VB63F); C- = Negative control (soy extract or skim milk, treated in same conditions of test samples, but after inoculation with saline solution (NaCl, 0.85%, w/v) in the place of bacterial strains. See **Table 3.1** for the description of strains.

This assay was performed based on the hypothesis that, if any of the tested strains presented proteolytic activity against soy proteins this strain would be selected and employed in the development of a probiotic fermented soy beverage. We hoped that during the fermentation the protein hydrolysis could lead to a reduction in the allergenicity of soy proteins and to the generation of bioactive peptides with, for example, antihypertensive and antioxidants potential. However, none of the evaluated strains presented proteolytic activity against milk and soy proteins, as can be seen in **Figure 3.1**. No reduction in the bands' intensity representing milk and soy proteins was observed for the samples treated with probiotic and *S. thermophilus* strains. Since the environments in which lactic acid bacteria grow are rich in several nutrients like sugars and proteins, most LAB are auxotrophic for a large number of amino acids and vitamins (Teusink & Molenaar, 2017). Thus, the results suggest that the tested strains used the putative free amino acids present in the milk and soy matrices, and therefore, did not require protein hydrolysis for growth. Song et al. (2008) studied the reduction of the immunoreactivity of commercial soybean products containing strains of *L. plantarum* CECT (ATCC 14917), *B. lactis* BB-12 and *Saccharomyces cereviseae* IFI 87. After SDS-PAGE of fermented samples, no proteolysis was observed, but the authors reported that all commercial fermented products showed very low immunoreactivity against soybean proteins.

3.1.3. *In vitro* carbohydrates fermentation

The population changes between 0 h (before incubation) and 24 h of incubation, for each strain grown in mMRS media supplemented with different carbohydrates, are presented in **Table 3.4.** Among the *Lactobacillus* strains grown in the presence of raffinose, *L. fermentum* PCC and *L. reuteri* RC-14 presented the highest population changes, followed by *L. acidophilus* LA-5. RC-14 presented the higher maximum growth rate (data not shown). The populations of *L. paracasei* F19 and *L. rhamnosus* LGG were not significantly different ($P>0.05$) from the negative control (described above, item 2.4). In a study using soluble soy extract, LeBlanc, Garro, & Savoy de Giori (2004) observed that *L. fermentum* strains presented high consumption of raffinose, with the great production of organic acids when the pH of the matrix was 5.5. Regarding *Bifidobacterium* strains, *B. longum* BB-46 presented the highest change in population in the presence of raffinose, while *B. longum* BB-02 population change after 24h of incubation was very similar to the negative control. Hou, Yu, and Chou (2000) verified that the fermentation of soy milk by *B. longum* BB-46 and *B. infantis* CCRC 14633 lead to a reduction in the concentrations of raffinose and stachyose. *S. thermophilus* TA-40 and TH-4 were able to grow in the presence of the majority of the tested carbohydrates, but *S. thermophilus* TH-4 presented the highest growth in mMRS with raffinose. Wang, Yu, Yang & Chou (2003) studied the effect of fermentation on the sugar contents of soymilk and observed that *S. thermophilus* CCRC 14085, alone or in combination with *Bifidobacterium* strains, presented a significant consumption of raffinose and stachyose, but *L. acidophilus* CCRC 14079 only showed a significant reduction of both carbohydrates when in combination with *Bifidobacterium* strains. In general, the tested *Bifidobacterium* and *Streptococcus thermophilus* strains did not grow or their growth was very similar to the negative control in the presence of the fructans tested. Among *Lactobacillus* strains, only F19 grew in the presence of all fructans (fructo-oligosaccharade-FOS, Inulins GR, and HP). Watson et al. (2013), studying the growth of several *Bifidobacterium* and *Lactobacillus* strains, also observed that BB-12 and the *B. longum* strains did not grow significantly in the presence of inulin and FOS.

Table 3.4. Changes (from 0 h to 24 h) in the populations of probiotic and starter cultures (log CFU/mL)[1] after the fermentation in mMRS containing different carbohydrates (see **Table 3.1** for description of strains).

Strains	Carbohydrates							
	Glucose	**Fructose**	**Sucrose**	**Lactose**	**Raffinose**	**FOS**	**Inulin-GR**	**Inulin-HP**
				Lactobacillus				
LA-5	3.95 (0.50)^{ABa}	4.02 (0.73)^{Aa}	3.64 (0.52)^{Aab}	2.82 (0.62)^{Dcd}	2.73 (0.52)^{BCcd}	3.18 (0.51)^{ABbcd}	3.38 (0.86)^{Aabc}	2.46 (0.83)^{BCd}
PCC	3.64 (0.66)^{BCa}	3.13 (0.44)^{Aab}	3.57 (0.50)^{Aa}	3.33 (0.19)^{BCa}	3.33 (0.83)^{ABa}	2.45 (0.98)^{CDbc}	2.04 (0.27)^{BCc*}	1.86 (0.69)^{Cc*}
RC-14	1.20 (1.07)^{Dc*}	1.25 (1.33)^{Bc*}	2.64 (0.09)^{Bab}	3.03 (0.27)^{Ba}	3.58 (0.42)^{Aa}	1.68 (0.47)^{Dbc}	1.47 (1.74)^{Cc}	1.36 (0.90)^{Cc*}
LGG	3.73 (0.60)^{ABCa}	3.15 (0.55)^{Aab}	1.10 (1.35)$^{Ce\#}$	1.25 (0.39)^{Ede*}	2.09 (0.49)^{Ccd*}	2.97 (0.68)^{ABCabc}	2.26 (1.06)^{ABCbc*}	3.00 (0.91)^{Babc}
GR-1	3.28 (0.46)^{Ca}	3.26 (0.55)^{Aa}	2.48 (0.29)^{Bb}	3.23 (0.01)^{ABa}	2.43 (0.54)^{Cb}	1.77 (0.13)^{Dc}	2.09 (0.25)^{BCbc}	2.37 (0.04)^{BCb}
F19	4.17 (0.58)^{Aa}	3.81 (0.64)^{Aab}	3.57 (0.31)^{Aabc}	3.35 (0.32)^{Abcd}	2.77 (0.27)^{BCd*}	3.88 (0.89)^{Aab}	3.10 (0.76)^{ABcd}	3.69 (0.38)^{Aabc}
LC-431	3.68 (0.46)^{ABCa}	3.13 (0.58)^{Ab}	2.41 (0.34)^{Bc}	3.32 (0.23)^{ABab}	2.57 (0.57)^{BCc}	2.49 (0.19)^{BCDc}	3.20 (0.12)^{ABab}	3.65 (0.75)^{Aab}
				Bifidobacterium				
BB-12	2.99 (0.15)^{Ba}	2.96 (0.10)^{Ba}	2.46 (0.11)^{Bb}	1.71 (0.17)^{Bd*}	2.41 (0.05)^{Bb}	2.37 (0.22)^{Bb}	2.03 (0.10)^{Ac}	1.97(0.24)^{Bc*}
BB-46	3.62 (0.16)^{Aa}	3.53 (0.10)^{Aa}	3.08 (0.11)^{Ab}	3.06 (0.18)^{Ab}	3.08 (0.12)^{Ab}	1.42 (0.08)$^{Cd\#}$	2.16 (0.08)^{Ac*}	2.29 (0.23)^{Ac*}
BB-02	3.08 (0.18)^{Bab}	2.87 (0.13)^{Bc}	2.92 (0.34)^{Abc}	3.13 (0.42)^{Aa}	2.40 (0.09)^{Bd*}	3.32 (0.02)^{Aa}	2.24 (0.14)$^{Ad\#}$	1.64 (0.15)$^{Ce\#}$
				Streptococcus thermophilus				
TH-4	2.88 (0.38)^{Acd}	2.72 (0.97)^{Acd}	3.55 (0.55)^{Aab}	2.64 (0.28)^{Acd*}	3.95 (1.16)^{Aa}	3.13 (0.63)^{Abc}	2.35 (0.57)^{Ad*}	2.74 (0.56)^{Acd}
ST-M6	1.57 (0.53)^{Bab*}	1.99 (0.19)^{Ba}	1.92 (0.37)^{Ba}	1.77 (0.18)^{Ba*}	0.47 (0.75)$^{Cc\#}$	1.65 (0.15)^{Bab*}	1.13 (0.41)^{Bb*}	1.93 (0.10)^{Ba}
TA-40	2.60 (0.29)^{Aab}	2.85 (0.38)^{Aa}	2.46 (0.63)^{Bab}	2.10 (0.10)^{ABb}	2.31 (0.63)^{Bab}	2.48 (0.32)^{Aab}	2.50 (0.19)^{Aab}	1.04 (0.49)^{Cc*}

$^{A-D}$ Different superscript capital letter in a column denote significant differences ($P<0.05$) between the *Lactobacillus* or *Bifidobacterium* or *Streptococcus thermophilus* strains for a same carbohydrate. $^{a-f}$ Different superscript lowercase letter in a row denote significant differences ($P<0.05$) for a same strain between the different carbohydrates. * Not significantly different ($P>0.05$) from the negative control (mMRS without the supplementation with carbohydrate). $^{\#}$ Significantly lower populations ($P<0.05$) than the negative control (mMRS without carbohydrate supplementation).

[1]Δpopulations = populations T24 (log CFU/mL) – populations T0 (log CFU/mL); T0 = initial populations (before incubation at 37 ºC); T24 = final populations (after incubation of 24 h at 37 ºC).

3.2. Technological and sensory acceptance of fermented soy beverages

3.2.1. Physical-chemical characteristics and fatty acid profile of fermented soy beverages

After screening of probiotic and starter cultures based on the ability of fermentation of raffinose and bile salt hydrolysis, *L. acidophilus* LA-5 and *B. longum* BB-46 presented deconjugation ability of a greater variety of bile salts, as well as a good growth in the presence of raffinose as also observed for *S. thermophilus* TH-4. Thus, these strains were employed, together with the acerola by-product, in the eight pilot-scale-marking formulations of FSB and their effect on technological and sensory characteristics of FSB was evaluated. The chemical composition of FSB is shown in **Table 3.5.** As expected, the moisture content was significantly influenced ($P<0.05$) by the presence of acerola by-product, since during the production of FSB4, FSB6, FSB7, and FSB8, a proportion of 2% of acerola by-product flour was added. The flour increased the dry matter content in these formulations, consequently reducing the moisture. However, the acerola by-product did not influence the fat, protein, and ash contents in whole samples ($P>0.05$). Previous results reported by Vieira et al. (2017) demonstrated that acerola by-product flour presented a low content of moisture and a high content of dietary fibre (~50%), which could explain the approximately 1% higher carbohydrate content and the reduction of moisture in FSB formulations added of this by-product. Espírito Santo et al. (2012) reported a similar reduction in moisture and increase in dietary fibre content in probiotic yoghurts added of fruit by-products when compared to the yogurt control formulation.

The fatty acid composition of different fermented soy beverages is shown **Table 3.6**. A chromatogram of one of the samples is shown in **Figure 3.2**. The presence of acerola by-product and of the probiotic strains did not influence the fatty acid profiles of the fermented soy beverages significant ($P>0.05$). Their fatty acid profiles showed the presence of the same fatty acids and in similar proportions as those reported by Dourtoglou et al. (2003) also for soy fermented products. The polyunsaturated fatty acid (PUFA) proportion was above 60% of the fatty acid composition for all FSB. In a meta-analysis of 60 selected trials, Mensink, Zock, Kester, and Katan (2003) reported that even small amounts of unsaturated fatty acids have an important effect on the ratio of total cholesterol/ HDL cholesterol. Linoleic acid was the more abundant fatty acid in all FSB, with concentrations of up to 55%. In the Dietary Reference Intake Report for Energy and Macronutrients published by the Institute of Medicine's Food and Nutrition Board defines an adequate intakes of linoleic acid as 17 g/d for men and 12 g/d for women, which corresponds to 5% to 6% of the diet energy for people between 19 and 50 years old (Food and Nutrition Board, Institute of Medicine, 2017). Thus, the consumption of a portion of 100 mL of FSB corresponds to approximately 8% of the daily needs of n-6 PUFA for men

and 11.5% for women. Johnson & Fritsche (2012) suggested that n-6 PUFA, including linoleic acid, decreases the risk of cardiovascular disease as indicated by current dietary recommendations. Therefore, the FSB showed to be a great source of essential fatty acids with potential for reducing the risk of cardiovascular diseases.

Table 3.5. The chemical composition of the different fermented soy beverages.

Fermented soy beverages[1]	Moisture (g/100 g)	Ash (g/100 g)	Protein (g/100 g)	Fat (g/100 g)	Total Carbohydrates[2] (g/100 g)
FSB1	79.67 (0.09)^{B}	0.69 (0.04)^{A}	3.65 (0.39)^{A}	2.32 (0.10)^{A}	13.67
FSB2	79.99(0.05)^{AB}	0.69 (0.02)^{A}	3.72 (0.10)^{A}	2.34 (0.11)^{A}	13.26
FSB3	79.96(0.16)^{AB}	0.71 (0.03)^{A}	3.64 (0.07)^{A}	2.34 (0.18)^{A}	13.35
FSB4	78.27(0.19)^{D}	0.68 (0.02)^{A}	3.64 (0,21)^{A}	2.41 (0.25)^{A}	15.00
FSB5	80.06(0.06)^{A}	0.66 (0.01)^{A}	3.60 (0,07)^{A}	2.43 (0.10)^{A}	13.25
FSB6	78.76(0.14)^{C}	0.70 (0.00)^{A}	3.71 (0.16)^{A}	2.47 (0.13)^{A}	14.36
FSB7	78.64(0.11)^{C}	0.68 (0.06)^{A}	3.78 (0.10)^{A}	2.57 (0.04)^{A}	14.33
FSB8	78.65(0.12)^{C}	0.69 (0.07)^{A}	3.73 (0.20)^{A}	2.54 (0.06)^{A}	14.39

Values are expressed as means (standard deviation). $^{A-D}$ Different superscript capital letters in a row denote significant differences ($P<0.05$) between fermented soy beverages.
[1] See **Table 3.2** for description of fermented soy beverages. [2] Total dietary fibres.

Table 3.6. Fatty acid profile of different fermented soy beverage

Fermented soy beverage[1]	Fatty acid profile (%)				
	Saturated		Unsaturated		
	C16:0	C18:0	C18:1 c9	C18:2 c9,c11	C18:3 c9,c12,c15
FSB1	11.16 (0.08)	3.88 (0.18)	23.91 (0.02)	55.20 (0.52)	5.87 (0.22)
FSB2	11.17 (0.04)	3.80 (0.37)	24.00 (0.09)	55.34 (0.10)	5.70 (0.03)
FSB3	11.22 (0.09)	3.84 (0.13)	24.21 (0.06)	55.10 (0.28)	5.65 (0.01)
FSB4	11.23 (0.12)	3.86 (0.18)	24.13 (0.25)	55.15 (0.56)	5.65 (0.01)
FSB5	11.17 (0.01)	3.75 (0.06)	23.98 (0.02)	55.43 (0.01)	5.68 (0.06)
FSB6	11.28 (0.11)	3.86 (0.00)	24.20 (0.00)	55.05 (0.06)	5.62 (0.03)
FSB7	11.40 (0.05)	3.88 (0.01)	24.24 (0.08)	54.89 (0.01)	5.60 (0.00)
FSB8	11.39 (0.06)	3.87 (0.05)	24.20 (0.08)	54.87 (0.06)	5.68 (0.01)

Values are expressed as mean % (standard deviation). No significant differences between the fermented soy beverages were observed (P>0.05). [1] See **Table 3.2** for description of fermented soy beverages.

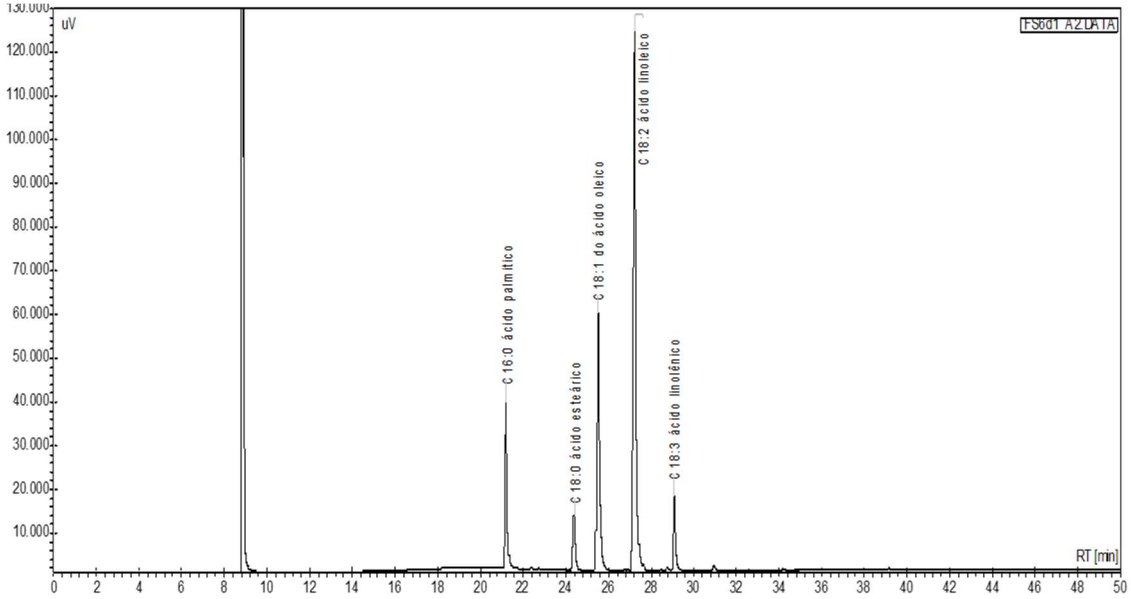


Figure 3.2. Chromatogram of the fatty acid profile of fermented soy beverage FSB6 (see **Table 3.2** for description of fermented soy beverage FSB6)

Figure 3.3. illustrate the evolution of titratable acidity of fermented soy beverages. The experimental design influenced the changes in lactic acid concentrations during refrigerated storage (28 days at 4 ºC) ($P<0.05$). The most significant factor in the titratable acidity increase was the presence of *B. longum* BB-46, followed closely by the presence of *L. acidophilus* and of acerola by-product. The time of storage was significant ($P<0.05$) for all fermented soy beverages, except for FSB1 and FSB4, both without any of the probiotic strains. Similarly, Donkor, Henriksson, Vasiljevic, & Shah, (2007c) observed that fermented soy prepared with a yoghurt culture and a probiotic co-culture (*L. acidophilus* L10, *B. animalis* B94 and *L. casei* L26) presented the highest increase in acidity when compared to fermented soy containing only the yoghurt culture. The FSB8, which presented all factors studied in the experimental design, had the highest titrable acidity values ($P<0.05$) among all the fermented soy beverages in almost all sampling periods, except for in the 21st day of storage, when it was the second more acid beverage. The greatest change between the first and the 28th day of storage was observed for FSB3 (0.61 g of lactic acid/100 g) and FSB5 (0.53 g of lactic acid/100 g), which presented *B. longum* BB-46 alone and in co-culture with *L. acidophilus* LA-5, respectively, but both without acerola by-product. Similarly, increases in lactic acid concentrations in synbiotic vegetable soy beverages between the first and 28th day of storage were reported by Battistini et al. (2018). İçier, Gündüz, Yilmaz, and Memeli, (2015) noted that probiotic soy beverages containing apple juice increased during the storage period and the beverage with 15% of apple juice presented the highest acidity. However, İçier et al. (2015) and

Espírito Santo et al. (2012) observed that, after 14 days of storage, the acidity did not change. We observed that co-cultures of BB-46 and LA-5 did not favor the viability of both strains, which may have influenced the higher production of lactic acid when the strains were isolated, only together with *S. thermophilus* TH-4 (data not shown).

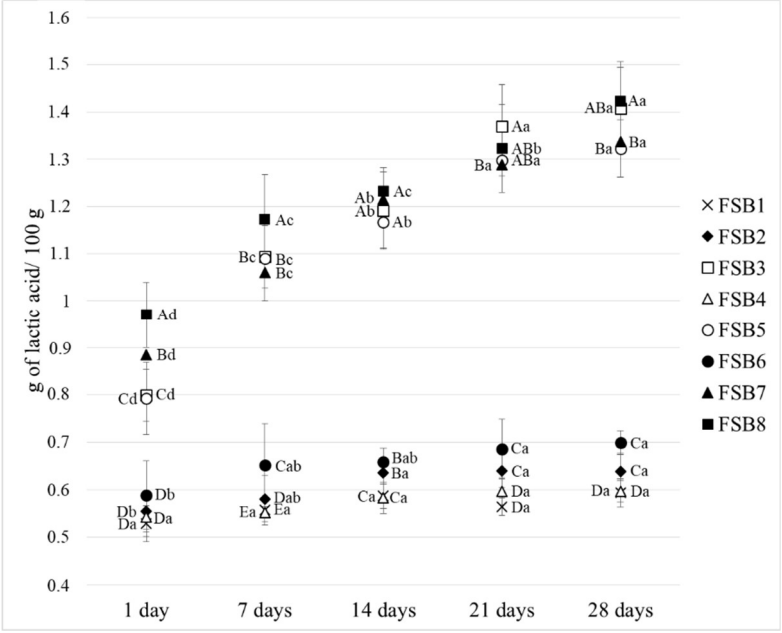


Figure 3.3. Evolution in the titratable acidity (g of lactic acid/100 g) observed for the fermented soy beverages during refrigerated storage (28 days at 4 ºC). See **Table 3.2** for the description of fermented soy beverages. [A-F] Different capital letters denote significant differences between fermented soy beverages for the same sampling period ($P<0.05$). [a-c] Different lowercase letters denote significant differences between different sampling periods of storage for the same fermented soy beverage ($P<0.05$).

3.2.2. Microbiological parameters and TPA of fermented soy beverages

Bacillus cereus, Salmonella spp. in 25 g, DNAse positive *Staphylococcus*, molds, and yeasts, as well as total coliforms and thermotolerant coliforms, were not detected in any of the FSB analyzed, indicating that all beverages were microbiologically safe for human consumption. Additionally, the population of *S. thermophilus* TH-4 ranged from 8 to 9 CFU/mL, and *L. acidophilus* LA-5 and *B longum* BB-46 ranged from 7 to 8 log CFU/mL during 28 days of refrigerated storage for all FSB (data not shown).

The instrumental texture profile of FSB during refrigerated storage is shown in **Table 3.7.** Each texture attribute was influenced significantly different ($P<0.05$) by the experimental design.

In general, the presence of acerola by-product increased significantly ($P<0.05$) the firmness and gumminess of the fermented soy beverages. Similarly, Perina et al. (2015) reported a significant influence of the presence of passion fruit peel-powder in the firmness of

probiotic yoghurts. Bedani et al. (2013) noted that the presence of okara flour (soybean by-product) contributed for significant changes in the texture profiles of the soy yogurt and the fibres present in this by-product might have contributed towards a higher firmness in soy yoghurt with okara. Pandey & Mishra (2015) observed that the presence of fructo-oligosaccharides (FOS) increased the hardness of synbiotic soy yoghurt significantly. The presence of *Lactobacillus acidophilus* LA-5 increased springiness significantly ($P<0.05$), whereas the presence of *Bifidobacterium longum* BB-46 increased gumminess for the fermented soy beverages significantly ($P<0.05$). Patrignani et al. (2018) reported a significant effect of *Bifidobacterium* strains on the rheology of fermented soy milk and a higher firmness and viscosity index were observed for the fermented soy milk in the presence of *B. aesculapii* FSM MRM 4.7 and a higher cohesiveness in the presence of *B. aeculapii* FSM MRM 4.6. The interaction of factors (acerola-by-product**L. acidophilus* LA-5**B. longum* BB-46) was significant ($P<0.05$) for firmness and gumminess and, in the presence of LA-5 and BB-46 without acerola by-product, the firmness and gumminess were significantly higher ($P<0.05$). Comparing the different sampling periods, we observed a significant variation ($P<0.05$) for all texture parameters, except for springiness that was only significant ($P<0.05$) when there was an interaction between the sampling periods and the other factors studied (sampling period*acerola by-product*LA-5*BB-46). Donkor, Henriksson, Singh, Vasiljevic, & Shah (2007b) noted that differences in storage were a reflection of gelation characteristic for the different soy yoghurts, in which raffinose and glucose may have influenced the metabolic activities of microorganism and affected the rheology of soy yoghurts.

3.2.3. Sensorial evaluation of fermented soy beverages

The mean values of acceptability scores (flavour, aroma, texture, appearance, and overall acceptance) for the fermented soy beverages studied are shown in **Table 3.8**. No significant difference ($P>0.05$) was observed between 7 and 21 days of refrigeration storage (4 ºC) for the different FSB for all the sensory attributes evaluated, thus the mean values were presented as general scores of each sensory attribute for each FSB. In general, the presence of *B. longum* BB-46 isolated or its combination with acerola by-product decreases the scores of flavour and overall acceptance FSB ($P<0.05$). The higher acidity previously described and the off-flavour caused by a small concentration of acetate (data not shown) for the fermented soy beverages which contained *B. longum* BB-46 may have negatively influenced the acceptability scores. In fact, Patrignani et al. (2018) observed that a probable production of acetic acid by *Bifidobacterium* resulting from the carbohydrates metabolism could affect the sensory

properties of fermented soymilk. Mondragón-Bernal, Alves, Teixeira, Ferreira, & Maurgeri Filho (2017) also noted that *Bifidobacterium* strains leaded to sensorial problems in synbiotic fermented soy product, which showed higher viable counts and pH below 4.0. Lower scores for flavour were observed for the fermented soy beverages FSB3, FSB5, FSB7, and FSB8, and the last two FSB had the lowest overall acceptance.

Additionally, the appearance was negatively influenced ($P<0.001$) by the presence of acerola by-product. Nonetheless, beverage FSB4 (with acerola by-product and without any probiotic strains) showed a higher frequency between "would certainly buy" and "would probably buy", which accounted together for 31.3% of the purchase intention (**Figure 3.4**). The correlation between the sensory attributes and the purchase intention was strongly uphill for the flavour and overall acceptance, and moderate uphill for the other attributes, as well as significant ($P<0.05$) for all attributes (Table 3.8). Studies, using passion-fruit by-product (Espírito-Santo et al., 2013) and okara (Bedani et al., 2013), did not show any significant influence of by-products in the appearance of milk and soy yoghurts, probably due to the light colouring of these by-products, which probably did not change the yoghurts colour, differently from what was here observed for the acerola by-product, which presented a brown dark colour.

Principal component analyses (PCA) was carried out to evaluate the relationship between the eight pilot-scale-making formulations (see Table 3.2) and the sensory attributes evaluations. **Figure 3.5**. illustrates the general scores of FSB and the sensory attributes. The first principal component (PC, F1) explained 72.02% of the total variance between FSB (72.02%) and the second component (F2) explained 24.53%, and both explained almost all the variability (96.56%) between FSB in relation to the sensory attributes. The sensory attributes were represented as vectors. The vectors that, when being decomposed in an axis (PC) are presented as a longer vector, better explain the variability among the by-product of that PC. Thus, the 72% of the variability explained in the first PC as due to the fact that these vary in relation to the five attributes evaluated (flavour, aroma, appearance, texture, and overall acceptance), with more emphasis on flavour. The 24.5% of the variability explained in the second PC are due to the fact that they vary mainly in relation to appearance and flavour attributes. In the PCA, each combination is located in the direction of the vector (attribute) that most characterizes it in relation to the other FSB.

Table 3.7. Instrumental texture profile (TPA) of the different fermented soy products studied during refrigerated storage at 4 ºC

Texture parameter	Time (days)	Fermented soy beverages[1]							
		FSB1	**FSB2**	**FSB3**	**FSB4**	**FSB5**	**FSB6**	**FSB7**	**FSB8**
Firmness (N)	**1**	0.147 (0.012)^{Ccd}	0.149 (0.008)^{Abcd}	0.144 (0.006)^{Ad}	0.162 (0.011)^{Bab}	0.151 (0.007)^{Bbcd}	0.164 (0.008)^{ABa}	0.160 (0.018)^{Babc}	0.161 (0.015)^{Aab}
	7	0.167 (0.008)^{ABa}	0.145 (0.009)^{Ab}	0.150 (0.018)^{Ab}	0.177 (0.023)^{Aa}	0.168 (0.033)^{Aa}	0.164 (0.009)^{ABa}	0.163 (0.023)^{Ba}	0.165 (0.012)^{Aa}
	14	0.171 (0.008)^{Aa}	0.148 (0.012)^{Ac}	0.155 (0.013)^{Abc}	0.147 (0.015)^{Cc}	0.174 (0.022)^{Aa}	0.172 (0.008)^{Aa}	0.154 (0.015)^{Bbc}	0.164 (0.012)^{Aab}
	21	0.157 (0.022)^{BCbc}	0.147 (0.016)^{Acde}	0.143 (0.011)^{Ade}	0.146 (0.014)^{Ccde}	0.171 (0.022)^{Aa}	0.154(0.019)^{BCbcd}	0.139 (0.005)^{Ce}	0.163 (0.005)^{Aab}
	28	0.158 (0.022)^{ABCb}	0.152 (0.013)^{Ab}	0.155 (0.012)^{Ab}	0.178 (0.010)^{Aa}	0.154 (0.018)^{Bb}	0.148 (0.016)^{Cb}	0.184 (0.013)^{Aa}	0.157 (0.015)^{Ab}
Cohesiveness	**1**	0.849 (0.100)^{Aa}	0.824 (0.097)^{Aab}	0.841 (0.101)^{Ba}	0.802 (0.053)^{Aab}	0.839 (0.081)^{Aa}	0.758 (0.016)^{Cb}	0.837 (0.081)^{Ba}	0.802 (0.049)^{Bab}
	7	0.818 (0.073)^{Aa}	0.841 (0.115)^{Aa}	0.852 (0.093)^{Ba}	0.812 (0.059)^{Aa}	0.851 (0.080)^{Aa}	0.821 (0.083)^{BCa}	0.842 (0.093)^{Ba}	0.815 (0.061)^{ABa}
	14	0.811 (0.069)^{Aa}	0.843 (0.104)^{Aa}	0.854 (0.104)^{Ba}	0.841 (0.105)^{Aa}	0.806 (0.047)^{Aa}	0.817 (0.073)^{BCa}	0.834 (0.095)^{Ba}	0.814 (0.065)^{ABa}
	21	0.835 (0.089)^{Ac}	0.857 (0.111)^{Abc}	0.935 (0.015)^{Aa}	0.834 (0.102)^{Ac}	0.872 (0.031)^{Aabc}	0.836 (0.101)^{Bc}	0.918 (0.021)^{Aab}	0.878 (0.012)^{Aabc}
	28	0.846 (0.108)^{Aab}	0.846 (0.101)^{Aab}	0.842 (0.099)^{Bb}	0.861 (0.020)^{Aab}	0.842 (0.086)^{Aab}	0.914 (0.030)^{Aa}	0.813 (0.062)^{Bb}	0.826 (0.064)^{ABb}
Springiness	**1**	0.996 (0.018)^{Aabc}	0.977 (0.037)^{Ac}	1.014 (0.048)^{Aa}	0.973 (0.029)^{Bc}	1.007 (0.025)^{Aab}	0.989 (0.036)^{Aabc}	0.980 (0.030)^{Bbc}	0.995(0.039)^{ABabc}
	7	0.988 (0.025)^{ABab}	1.000 (0.032)^{Aab}	0.996 (0.023)^{ABab}	0.979 (0.042)^{Bb}	0.983 (0.024)^{Ab}	0.995 (0.028)^{Aab}	1.014 (0.028)^{Aa}	0.993 (0.028)^{Bab}
	14	0.979 (0.036)^{ABbc}	1.000 (0.035)^{Aab}	0.971 (0.039)^{Bc}	0.979 (0.034)^{ABbc}	0.979 (0.028)^{Abc}	0.977 (0.029)^{Abc}	0.977 (0.039)^{Bbc}	1.008 (0.048)^{ABa}
	21	0.965 (0.034)^{Bb}	0.986 (0.036)^{Aab}	0.998 (0.018)^{Aba}	1.006 (0.025)^{Aa}	0.987 (0.012)^{Aab}	1.006 (0.025)Aa	0.986 (0.018)^{ABab}	0.981 (0.012)^{Bab}
	28	0.978 (0.032)^{ABb}	1.005 (0.037)^{Aab}	0.989 (0.017)^{ABb}	0.990 (0.019)^{ABb}	0.988 (0.021)^{Ab}	0.991 (0.017)^{Ab}	0.979 (0.036)^{Bb}	1.023 (0.074)^{Aa}
Gumminess (N)	**1**	0.124 (0.009)^{Bab}	0.122 (0.011)^{Aab}	0.121 (0.014)^{Bb}	0.130 (0.029)^{BCab}	0.126 (0.010)^{Cab}	0.134 (0.011)^{ABa}	0.134 (0.015)^{Ba}	0.130 (0.020)^{Bab}
	7	0.137 (0.017)^{Aa}	0.121 (0.010)^{Ab}	0.126 (0.008)^{ABb}	0.144 (0.042)^{Aa}	0.141 (0.019)^{Aa}	0.135 (0.018)^{ABa}	0.135 (0.012)^{Ba}	0.135 (0.019)^{ABa}
	14	0.139 (0.015)^{Aa}	0.124 (0.011)^{Ab}	0.132 (0.013)^{ABab}	0.122 (0.034)^{BCb}	0.141 (0.025)^{ABa}	0.141 (0.014)^{Aa}	0.126 (0.009)^{Bb}	0.134 (0.020)^{ABab}
	21	0.130 (0.006)^{ABbc}	0.123 (0.008)^{Abc}	0.134 (0.010)^{Ab}	0.121 (0.025)^{Cc}	0.148 (0.015)^{Aa}	0.128 (0.006)^{Bbc}	0.132 (0.011)^{Bbc}	0.143 (0.005)^{Aab}
	28	0.132 (0.009)^{ABb}	0.128 (0.016)^{Ab}	0.130 (0.011)^{ABb}	0.134 (0.019)^{ABb}	0.128 (0.013)^{BCb}	0.135 (0.012)^{ABb}	0.150 (0.008)^{Aa}	0.130 (0.017)^{Bb}

$^{A-D}$ Different superscript capital letter in a column denote significant differences ($P<0.05$) between the different storage time for the same fermented soy beverage.
$^{a-f}$ Different superscript lowercase letter in a row denote significant differences ($P<0.05$) between the different fermented soy beverage for the same storage time.
Values are expressed as mean (SD). [1]See **Table 3.2** for the description of fermented soy products. [2]Values expressed in the module.

Table 3.8. Scores of sensory parameters (Overall acceptance, Appearance, Aroma, Flavour, and Texture) and the correlation between each parameter and purchase intention obtained for the different fermented soy beverage.

Fermented soy beverage[1]	Overall acceptance	Appearance	Aroma	Flavour	Texture
FSB1	5.63 (2.16)^{a}	5.69 (2.32)^{a}	5.91 (2.03)^{a}	5.43 (2.50)^{ab}	5.59 (2.77)^{a}
FSB2	5.74 (2.22)^{a}	5.45 (2.41)^{a}	5.88 (2.39)^{a}	5.70 (2.54)^{ab}	5.37 (2.50)^{a}
FSB3	5.15 (2.22)^{ab}	5.71 (2.35)^{a}	5.30 (2.31)^{ab}	4.87 (2.77)^{bc}	5.61 (2.56)^{a}
FSB4	5.88 (2.30)^{a}	4.58 (2.49)^{b}	5.67 (2.70)^{ab}	6.06 (2.57)^{a}	5.41 (2.72)^{a}
FSB5	5.44 (2.27)^{ab}	5.63 (2.47)^{a}	5.16 (2.65)^{ab}	4.89 (2.77)^{bc}	5.24 (2.78)^{a}
FSB6	5.42 (2.39)^{ab}	4.74 (2.68)^{b}	5.36 (2.24)^{ab}	5.58 (2.75)^{ab}	5.23 (2.90)^{a}
FSB7	4.61 (2.74)^{b}	4.64 (2.56)^{b}	5.04 (2.62)^{ab}	4.20 (2.62)^{c}	4.93 (2.62)^{a}
FSB8	4.60 (2.51)^{b}	4.10 (2.50)^{b}	4.68 (2.52)^{b}	4.28 (2.85)^{c}	4.71 (2.51)^{a}
r-	0.766*	0.484*	0.489*	0.761*	0.487*

Overall mean for 7 and 21 days of refrigerated storage of fermented soy beverage (SD) $^{a-c}$ Different superscript lowercase letter in a row denote significant differences ($P<0.05$) between the different fermented soy beverages. *r*- Correlation between purchase intent and each sensorial parameter. * Significant ($P<0.05$) correlation. [1]See **Table 3.2** for the description of fermented soy products.

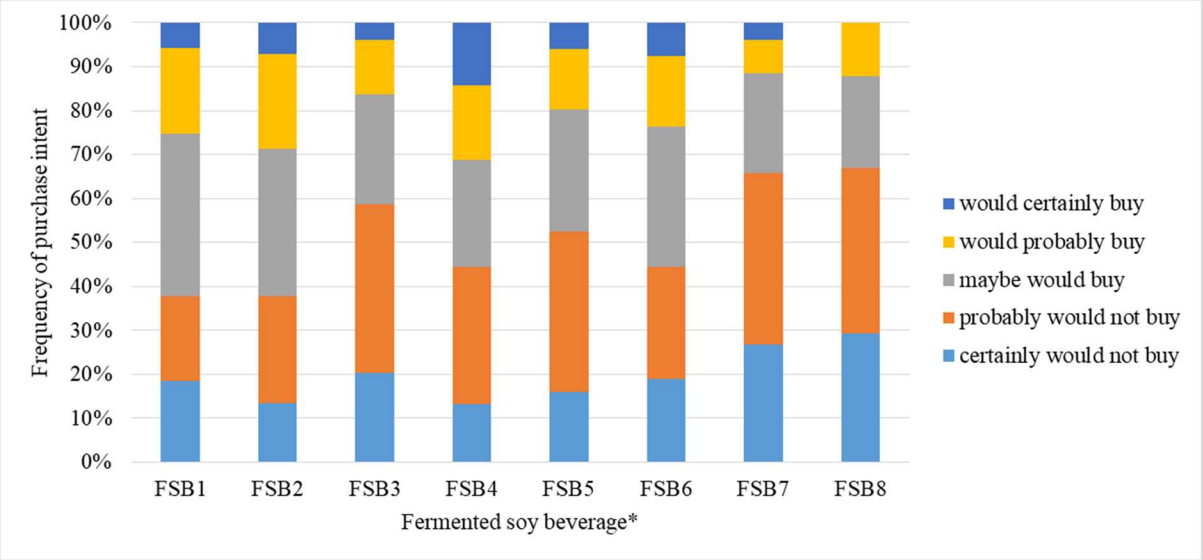


Figure 3.4. The frequency of purchase intention (%) for different fermented soy beverages. *See **Table 3.2.** for fermented soy beverage description.

The beverages FSB7 and FSB8 were less characterized by all five sensory attributes evaluated, indicating that the presence of *B. longum* BB-46 and acerola by-product, combined or not with *L. acidophilus* LA-5, were less accepted regarding flavour, aroma, texture, and appearance. On the other hand, FSB1 and FSB2 were more accepted in relation to flavour,

aroma, texture, and appearance. These FSB represented the samples with only *L. acidophilus* LA-5 (FSB2) and in the absence of the three factors evaluated (FSB1). FSB4 was more characterized by the flavour attribute and less characterized by the appearance attribute (opposite direction considering PC2). The overall acceptance of this combination was also good, as were the combinations FSB1 and FSB2. The FSB3 (*B. longum* BB-46) and FSB5 (*B. longum* BB-46 + *L. acidophilus* LA-5) combinations were very similar in relation to the sensory attributes, with positive prominence for the appearance attribute, as well as the combination FSB1 which is absent of any probiotic or the acerola by-product. The FSB6 combination was not specifically highlighted by any sensory attributes when compared to other combinations. The lower scores of acceptability of similar products were reported for several authors (Andrés, Tenorio, & Villanueva, 2015, Andrés, Villanueva, & Tenorio, 2016, Bedani et al., 2013, 2014, Patrignani et al., 2018).

We dismembered the results to verify the effect of the storage period (7 and 21 days) on the acceptability of fermented soy beverages (**Figure 3.6.**). The first component (PC1-F1) explained most of the variability between combinations (71%) and the second component (PC2-F2) accounted for 20 %. Clearly, it can be noted that consumer acceptance was lower after 21 days of storage, mainly for the fermented soy beverages FSB4, FSB6 e FSB7, thus indicating a difference that was not detected when performing the analysis of variance. The 21 days of storage has a negative impact on the attributes evaluates when compared to the 7 days. The beverage FSB8 already had a bad result on 7 days. Bedani et al. (2013, 2014) attributed the lower scores of acceptability for the fermented soy products to the volunteers' lack of habit to consume soy-based products. Additionally, studies have pointed that health benefits claims influenced positively the acceptance of soy products. Russell, Drake, & Gerard (2006) noted that health claims as "develops and maintains healthy bones", "decrease chances of heart disease", and "contains no animal fat" or "does not contain genetically modified ingredients" attributed to consumption of protein bars from whey and soy increased their acceptance significantly. A similar increase in acceptance was reported by Drake and Gerard (2003) for the soy-fortified yoghurts. On the other hand, Teh, Dougherty, and Camire, (2007) showed that only nutritional information did not affect acceptability scores of frozen soy products. In view of this, further studies should be conducted to assess whether other concentrations of acerola by-product are able to attenuate the disagreeable colour, which, according to the volunteers' opinion.

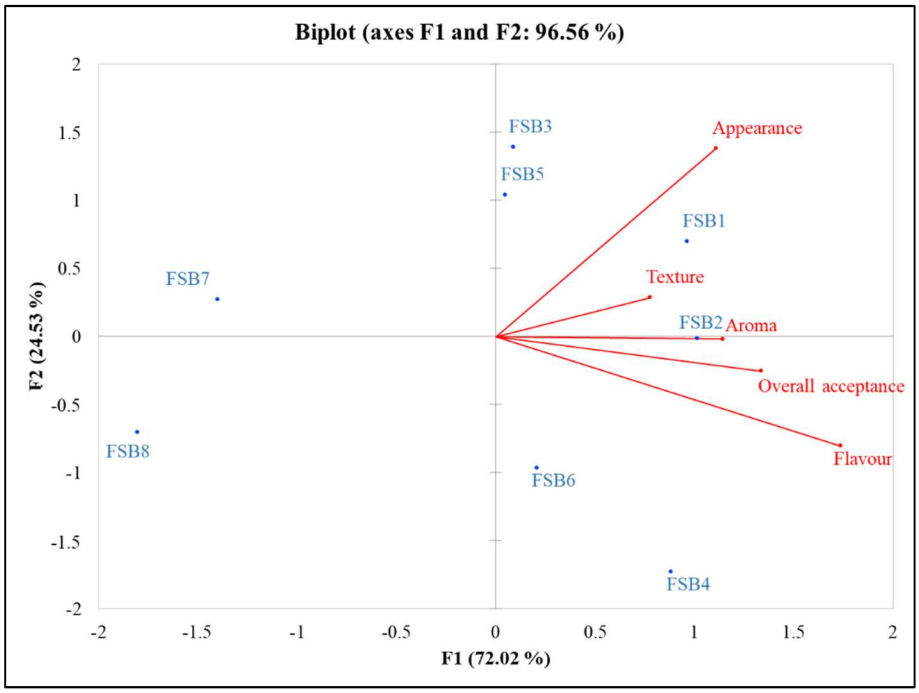


Figure 3.5. Principal Component graph of fermented soy beverages (blue) for the sensorial parameters (red). See **Table 3.2** for the description of fermented soy beverages.

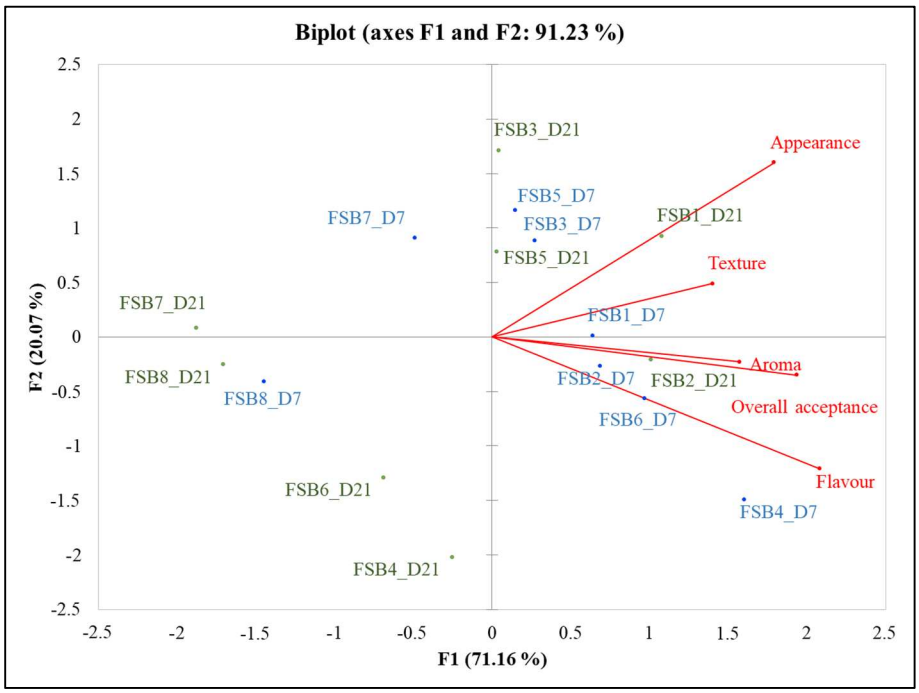


Figure 3.6. Principal Component graph of fermented soy beverages after 7 days (blue) and 21 days (green) of refrigeration storage (4 ºC) for the sensorial parameters (red). See **Table 3.2** for the description of fermented soy beverages.

4. CONCLUSION

The current study showed that, even though none of the strains tested presented proteolytic ability, *L. acidophilus* LA-5 and *B. longum* BB-46 were able to deconjugate more types of bile salts and grew well in the presence of raffinose. In addition, *S. thermophilus* TH-

4 grew better in the presence of raffinose among the *S. thermophilus* strains evaluated. The fermented soy beverages were significantly ($P<0.05$) influenced by the experimental design, with a higher dry matter content, an increased firmness and a lower sensory acceptance observed for the FSB containing the acerola by-product. The low sensory acceptance of the beverages with *B. longum* may have resulted from the high acidity and the off-flavour provided by the presence of small concentrations of acetate produced by this strain during the fermentation process and the refrigeration storage (post-acidification). The brown aspect for the beverages with acerola by-product influenced towards the low scores for appearance obtained for these soy beverages. On the other hand, the FSB4 with acerola by-product and without any probiotic strains showed the highest frequency of "would certainly buy" and "would probably buy" of purchase intention.

5. REFERENCES

American Public Health Association. (2001). *Compendium of methods for the microbiological examination of foods.* In F.P. DOWNES, K. ITO (eds), 4th ed., 2001, 676p.

André, V., Tenorio, M.-D., & Villanueva, M.-J. (2015). Sensory profile, soluble sugars, organic acids, and mineral content of milk- and soy-juice based beverages. *Food Chemistry, 173*, 1100-1106.

André, V., Villanueva, M.-J., & Tenorio, M.-D. (2016). Influence of high pressure processing on microbial shelf life, sensory profile, soluble sugars, organic acids, and mineral content of milk- and soy-smoothies. *LWT – Food Science and Technology, 65*, 98-105.

AOAC. (1984). *Official Methods of Analysis*. (14th ed.). Washington, DC: Association of Official Analytical Chemists.

Battistini, C., Gullón, B., Ichimura, R.S., Gomes, A.M.P., Ribeiro, E.P., Kunigk, L., et al. (2018) Development and characterization of an innovative synbiotic fermented beverage based on vegetable soybean. *Brazilian Journal of Microbiology*, 49(2), 303-309.

Bedani, R., Campos, M.M.S., Castro, I.A., Rossi, E.A., & Saad, S.M.I. (2013). Incorporation of soybean by-product okara and inulin in a probiotic soy yoghurt: texture profile and sensory acceptance. *Journal of the Science of Food and Agriculture, 94(1),* 119-125.

Bedani, R., Rossi, E.A., Cavallini, D.C.U., Pinto, R.A., Vendramini, R.C., Augursto, E.M., et al. (2015). Influence of daily consumption of synbiotic soy-based product supplemented with okara soybean by-product on risk factors for cardiovascular diseases. *Food Research International*, *73*, 142-148.

Bedani, R., Vieira, A.D.S., Rossi, E.A., & Saad, S.M.I. (2014). Tropical fruit pulp decreased probiotic survival to in vitro gastrointestinal stress in synbiotic soy yoghurt with okara during storage. *LWT – Food Science and Technology*, *55*, 436-443.

Bicola, V., Tulini, F.L., Choiset, Y., Rabesona, H., Ivanova, I., Chobert, J.-M., et al. (2016). Proteolytic activity of *Enterococcus faecalis* VB63F for reduction of allergenicity of bovine milk proteins. *Journal of Dairy Science, 99(7)*, 5144-5154.

Bligh, E.G. & Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37(8)*, 911-917.

Blomqvist, T., Steinmoen, H., & Håvarstein, L. S. (2006). Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*. *Applied and Environmental Microbiology*, *72*, 6751-6756.

Brashears, M.M., Gilliland, S.E., & Buck, L.M. (1998). Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. *Journal of Dairy Science, 81,* 2103-2110.

Buriti, F. C. A., Cardarelli, H. R., & Saad, S. M. I. (2007). Biopreservation by *Lactobacillus paracasei* in coculture with *Streptococcus thermophilus* in potentially probiotic and synbiotic fresh cream cheeses. *Journal of Food Protection*, *70*, 228-235.

Buriti, F.C.A., Castro, I.A., & Saad, S.M.I. (2010). Effect of refrigeration, freezing and replacement of milk fat by inulin and whey protein concentrate on texture profile and sensory acceptance of synbiotic guava mousses. *Food Chemistry, 123*, 1190-1197.

Chand, D., Avinash, V.S., Yadav, Y., Pundle, A.V., Suresh, C.G., & Ramaamy, S. (2017). Molecular features of bile salt hydrolases and relevance in human health. *Biochimica et Miophysica Acta*, *1861,* 2981-2991.

Da Silva, J.K., Cazarin, C.B.B., Batista, A.G., & Maróstica Jr., M. (2014). Effect of passion-fruit (*Passiflora edulis*) byproduct intake in antioxidant status of Wistar rats tissues. *LWT – Food Science and Technology*, *59*, 1213-1219.

De Lacey, A.M.L., Pérez-Santín, E., López-Caballero, M.E., & Montero, P. (2014). Survival and metabolic activity of probiotic bacteria in green tea. *LWT – Food Science and Technology*, *55*, 314-322.

Do Espírito Santo, A.P., Cartolano, N.S., Silva, T.F., Soares, F.A.S.M., Gioielli, L.A., Perego, P., et al. (2012). Fibers from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. *International Journal of Food Microbiology, 154*, 135-144.

Donkor, O.N., Henriksson, A., Singh, T.K., Vasiljevic, T., & Shah, N.P. (2007a). ACE-inhibitoty activity of probiotic yoghurt. *International Dairy Journal, 17*, 1321-1331.

Donkor, O.N., Henriksson, A., Vasiljevic, T., & Shah, N.P. (2007b). Rheological properties and sensory characteristics of set-type soy yogurt. *Journal of Agricultural and Food Chemistry, 55*, 9868-9876.

Donkor, O.N., Henriksson, A., Vasiljevic, T., & Shah, N.P. (2007c). α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk, *Food Chemistry, 104*, 10-20.

Dourtoglou, V.G., Dourtoglou, Th., Antonopoulos, A., Stefanou, E., Lalas, S., & Poulos, C. (2003). Deterction of olive oil adulteration using principal component analysis applied on total and region FA content. *Journal of the American Oil Chemists' Society, 80 (3),* 203-208.

Drake, M.A., & Gerard, P.D. (2003). Consumer attitudes and Acceptability of Soy-fortified Yogurts. *Journal of Food Science, 68(3),* 1118-1122.

Duzzioni, A.G., Lenton, V.M., Silva, D.I.S., & Barrozo, M.A.S. (2013). Effect of drying kinetics on main bioactive compounds and antioxidant activity of acerola (*Malpighia emarginata* D.C.) residue. *International Journal of Food Science and Technology*, *48*, 1041-1047.

El-Ghaish, S., Dalgalarrondo, M., Choiset, Y., Sitohy, M., Ivanova, I., Haertlé, T., et al. (2010). Screening of strains of lactococci isolated from Egyptian dairy products for their proteolytic activity. *Food Chemistry*, *120,* 758-764.

Espírito-Santo, A.P., Lagazzo, A., Sousa, A.L.O.P., Perego, P., Converti, A., & Oliveira, M.N. (2013). Rheology, spontaneous whey separation, microstructure and sensorial characteristics of probiotic yoghurts enriched with passion fruit fiber. *Food Research International, 50,* 224-231.

Farnworth, E.R., Mainville, I., Desjardins, M.-P., Gardner, N., Fliss, I., & Champagne, C. (2007). Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. *International Journal of Food Microbiology, 116*, 174– 181.

Food and Nutrition Board, Institute of Medicine. (2002). Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids. Washington, DC: National Academies Press. http://books.nap.edu/catalog.php?record_id=10490. Accessed 15 December 2017.

Fukuda, M., Kobayashi, M., & Honda, Y. (2017). Functional component and health benefits of fermented soymilk. In A. M. Grumezescu, & A. M. Holban (Eds.), *Soft chemistry and food fermentation*. (pp. 145-178), Handbook of Food Bioengineering (3rd volume). London: Academic Press.

Galanakis, C.M. (2013). Emerging technologies for the production of nutraceuticals from agricultural by-products: A viewpoint of opportunities and challenges. *Food and Bioproducts Processing, 91,* 573-579.

García-Cayuela, T., Díez-Municio, M., Herrero, M., Martínez-Cuestam M.C., Peláez, C., Requena, T., et al. (2014). Selective fermentation of potential prebiotic lactose-derived oligosaccharides by probiotic bacteria. *International Dairy Journal*, *38*, 11-15.

Hartman, L., & Lago, R.C.A. (1973). Rapid preparation on fatty acid methyl esters form lipids. *Laboratory Practice*, 22(6), 475-476.

Hou, J.-W., Yu, R.-C., & Chou, C.-C. (2000). Changes in some components of soymilk during fermentation with bifidobacteria. *Food Research International, 33*, 393-397.

IAL (2008). Analytical standards of the Instituto Adolfo Lutz: physicochemical methods for food analysis.http://www.ial.sp.gov.br/resources/editorinplace/ial/2016_3_19/analisedealimentosial_2008.pdf/Accessed 15.02.2016.

İçier, F., Gündüz, G.T., Yilmaz, B., & Memeli, Z. (2015). Changes on some quality characteristics of fermented soy milk beverage with added apple juice. *LWT – Food Science and Technology, 63*, 57-64.

IDF (1995). Fermented and non-fermented milk products. Detection and enumeration of *Lactobacillus acidophilus*. Culture media. FIL-IDF Standard 306. International Dairy Federation, Brussels.

Johnson, G.H., & Fritsche, K. (2012). Effect of dietary linoleic acid on markers of inflammation in healthy persons: A systematic review of randomized controlled trials. *Journal of the Academy of Nutrition and Dietetics, 112(7)*, 1029-1041.

Joyce, S.A., MacSharry, J., Casey. P.G., Kinsella, M., Murphy, E.F., Shanaham, F., et al. (2014). Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. *PNAS*, *111(20*), 7421-7426.

Kowalska, H., Czajkowska, K., Cichowska, J., & Lenart, A. (2017). What's new in biopotential of fruit and vegetable by-products applied in the food processing industry? *Trends in Food Science & Technology,* 67, 150-159.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680-685.

LeBlanc, J.G., Garro, M.S., & Savoy de Giori, G. (2004). Effect of pH on *Lactobacillus fermentum* growth, raffinose removal, α-galactosidase activity and fermentation products. *Applied Microbial and Cell Physiology, 65*, 119-123.

Lee, S.Y. & Hur, S.J. (2017). Anthypertensive peptides from animal products, marine organisms, and plants. *Food Chemistry, 228*, 506-517.

Liong, M.T., & Shah, N.P. (2005a) Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. *International Dairy Journal, 15,* 391-398.

Liong, M.T., & Shah, N.P. (2005b) Bile salt deconjugation and BSH acitivity of five bifidobacterial strain and their cholesterol co-precipitating properties. *Food Research International*, 38, 135-142.

Liong, M.-T., Easa, A.M., Lim, P.-T., & Kang, J.-Y. (2009). Survival, growth characteristics and bioactive potential of Lactobacillus acidophilus in a soy-based cream cheese. *Journal of the Science of Food and Agriculture, 89*, 1382-1391.

Liu, M., Bayjanov, J.R., Renckens, B., Nauta, A., & Siezen, R.J. (2010). The proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BMC Genomics, 11(36),* 1-15.

Marco, M.L., Heeney, D., Binda, S., Cifelli, C.J., Cotter, P.D., Foligné, B., et al. (2017). Health benefits of fermented foods: microbiota and beyond. *Current Opinion in Biotechnology*, *44*, 94-102.

Mensink, R.P., Zock, P.L., Kester, A.D.M., & Katan, M. (2003). Effect of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *The American Journal of Clinical Nutrition, 77,* 1146-1155.

Miremadi, F., Ayyash, M., Sherkat, F., & Stojanovska, L. (2014) Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic lactobacilli and bifidobacteria. *Journal of Functional Foods, 9,* 295-305.

Mohammed, M. (2011). Acerola (*Malpighia emarginata* DC.). In E.M. YAHIA, (Ed.), *Postharvest biology and technology of tropical and subtropical fruits: Açai to citrus* (pp. 27-48). Oxford: Woodhead Publishing Limited.

Mondragón-Bernal, O.L., Alves, J.G.L.F., Teixeira, M.A., Ferreira, M.F.P., Maugeri Filho, F. (2017). Stability and functionality of synbiotic soy food during shelf life. *Journal of Functional Foods,* 35, 134-145.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from Sour milk. *Journal of Dairy Science,* 78, 777-783.

Pandey, S.M., & Mishra, H.N. (2015). Optimization of the prebiotic & probiotic concentration and incubation temperature for the preparation of synbiotic soy yogurt using response surface methodology. *LWT – Food Science and Technology, 62,* 458-467.

Patrignani, F., Modesto, M., Michelini, S., Sansosti, M.C., Serrazanetti, D.I., Qvirist, L. et al. (2018). Technological potential of *Bifidobacterium aesculapii* strains for fermented soymilk production. *LWT – Food Science and Technology, 89,* 689-696.

Pavlović, N., Stankov, K., & Mikov, M. (2012). Probiotic – Interactions with bile acids and impact on cholesterol metabolism. *Applied Biochemistry and Biotechnology*, *168,* 1880-1895.

Paz, M., Gúllon, P., Barroso, M.F., Carvalho, A.P., Domingues, V.F., Gomes, A.M., et al. (2015). Brazilian fruit pulp as functional foods and additives: Evaluation of bioactive compounds. *Food Chemistry, 172*, 462-468.

Pereira, D.I.A., McCartney, A.L., & Gibson, G.R. (2003). An in vitro study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. *Applied and Environmental Microbiology, 69(8),* 4743-4752.

Perina, N.P., Granato, D., Hirota, C., Cruz, A.G., Bogsan, C.S.B., & Oliveira, M.N. (2015). Effect of vegetable-oil emulsion and passion fruit peel-powder on sensory acceptance of functional yogurt. *Food Research International, 70*, 134-141.

Pihlanto, A., & Korhonen, H. (2015). Bioactive peptides from fermented foods and health promotion. In W. Holzapfel (Ed.), *Advanced in Fermented Foods and Beverages*. (pp. 39-74) Amsterdam: Woodhead Publishing Series in Food Science, Technology and Nutrition.

Richter, R.L., & Vedamuthu, E.R. (2001). *Compendium of the methods for the microbiological examination of foods*, 4th ed. p.483–495. Washington, DC: APHA.

Rivera-Espinoza, Y., & Gallardo-Navarro, Y. (2010). Non-dairy probiotic products. *Food Microbiology*, *27,* 1-11.

Rochette, N.F.G., Mota, E.F., Nunes-Pinheiro, D.C.S., Bezerra, C.F., de Oliveira, M.L.M., da Silva, A.C.M., et al. (2013). Effect of the pretreatment with acerola (*Malpighia emarginata* DC.) juice on ethanol-induced oxidative stress in mice – Hepatoprotective potential of acerola juice. *Free Radicals and Antioxidants, 3*, S16-S21.

Russell, T.A., Drake, M.A., & Gerard, P.D. (2006). Sensory properties of whey and soy proteins. *Journal of Food Science, 71(6)*, S447-S455.

Ryu, S. I., Kim, B. G., Park, M. S., Lee, Y. B., & Lee, S. B. (2007). Evaluation of enhanced hygroscopicity, bifidogenicity, and anticariogenicity of enzymatically synthesized β-galactosyl-trehalose oligosaccharides. *Journal of Agricultural and Food Chemistry*, *55*, 4184-4188.

Sanjukta, S. & Rai, A.K. (2016). Production of bioactive peptides during soybean fermentation and their potential health benefits. *Trends in Food Science & Technology*, *50*, 1-10.

Santos, K.M.O., Vieira, A.D.S., Buriti, F.C.A., Nascimento, J.C.F., Melo, M.E.S., Bruno, L.M. et al. (2015). Artizanal coalho cheeses as source of beneficial *Lactobacillus plantarum* and *Lactobacillus rhamnosus* strains. *Dairy Science & Technology*, 95, 209-230.

Shehata, M.G., El Sohaimy, S.A., El-Sahn, M.A., & Youssef, M.M. (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Annals of Agricultural Science*, *61(1),* 65-75.

Singh, B.P., & Vij, S. (2017). Growth and bioactive peptides production potential of *Lactobacillus plantarum* strain C2 in soy milk: A LC-MS/MS based revelation for peptides biofunctionality. LWT-*Food Science and Technology*, *86*, 293-301.

Song, Y.-S., Frias, J., Martinez-Villaluenga, C., Vidal-Valdeverde, C., & Gonzalez de Mejia, E. (2008). Immunoreactivity reduction of soybean meal by fermentation, effect on amino acid composition and antigenicity of commercial soy products. *Food Chemistry, 108,* 571-581.

Sridevi, N., Vishwe, P., & Prabhune, A. (2009). Hypocholesteremic effect of bile salt hydrolase from *Lactobacillus buchneri* ATCC 4005. *Food Research International*, *42*, 516-520.

Tanaka, H., Doesburg, K., Iwasaki, T., & Mierau, I. (1999). Screening of lactic acid bacteria for bile salt hydrolase activity. *Journal of Dairy Science, 82*, 2530-2535.

Teh, T., Dougherty, M.P., & Camire, M.E. How do consumer attitudes influence acceptance of a NovelWild Blueberry-Soy Product? *Journal of Food Science, 72(7),* S516-S521.

Teusink, B., & Molenaar, D. (2017). Systems biology of lactic acid bacteria: For food and thought. *Current Opinion in Systems Biology, 6*, 7-13.

Ugarte, M.B., Guglielmotti, D., Giraffa, G., Reinheimer, J., & Hynes, E. (2006). Nonstarter lactobacilli isolated from soft and semihard Argentinean cheeses: genetic characterization and resistance to biological barriers. *Journal of Food Protection, 69(12),* 2983-2991.

Vieira, A.D.S., Bedani, R., Albuquerque, M.A.C., Biscola, V., & Saad, S.M.I. (2017). The impact of fruit and soybean by-products and amaranth on the growth of probiotic and starter microorganisms. *Food Research International, 97*, 356-363.

Vinderola, C. G., Prosello, W., Ghiberto, D., & Reinheimer, J. A. (2000). Viability of probiotic (*Bifidobacterium, Lactobacillus acidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in Argentinian fresco cheese. *Journal of Dairy Science*, *83*, 1905–1911.

Vinderola, G., Burns, P., & Reinheimer, J. (2017). Probiotics in Nondairy Products. In F. Mariotti (Ed), *Vegetarian and plant-based diets in health and disease prevention* (pp. 809-835). London: Academic Press.

Wang, Y.-C., Yu, R.-C., Yang, H.-Y., & Chou, C.-C. (2003). Sugar and acid contents in soymilk fermented with lactic acid bacteria alone or simultaneously with bifidobacteria. *Food Microbiology, 20*, 333-338.

Watson, D., Motherway, M.O.C., Schoterman, M.H.C., Neerven, R.J.J.V., Nauta, A., & Sinderen, D.V. (2012). Selective carbohydrate utilization by lactobacilli and bifidobacteria. *Journal of Applied Microbiology, 114,* 1132-1146.

Yeo, S.-K., & Liong, M.-T. (2010). Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics. *International Journal of Food Science and Nutrition, 61(2),* 161-181.

Chapter 4

Effect of acerola by-product added to a probiotic fermented soy beverage on survival of Bifidobacterium longum and Lactobacillus acidophilus in simulated gastrointestinal tract, evaluated during refrigerated storage.

Manuscript to be submitted for publication as:

VIEIRA, A.D.S.; PADILHA, M.; BEDANI, R.; SAAD, S.M.I. *Effect of acerola by-product added to a probiotic fermented soy beverage on survival of Bifidobacterium longum and Lactobacillus acidophilus in simulated gastrointestinal tract, evaluated during refrigerated storage.*

ABSTRACT

The effect of acerola by-product (ABP), *Lactobacillus acidophilus* LA-5, and *Bifidobacterium longum* BB-46 added during production of a fermented soy beverage (FSB) on probiotic viability and on probiotic survival in the product submitted to *in vitro* simulatied gastrointestinal (GI) conditions during 28 days of storage at 4 ºC were investigated. Eight trials of FSB were studied, using a factorial 2^3 design, in which the variable evaluated were *X1*-LA-5, *X2*-BB-46, and *X3*-ABP, in two levels (presence or absence). The 8 products, were prepared with water-soluble soy extract, and fermented with *Streptococcus thermophilus* TH-4. Enumeration of microorganisms was determined employing quantitative Real-Time PCR (qPCR) combined with PMA treatment (PMA-qPCR). Probiotic viable populations ranged from 7.0 to 8.2 log CFU equivalent/mL for 28 days, and the presence of the co-culture (LA-5+BB-46) and of the ABP did not affect the viability of both probiotics significantly ($P<0.05$). However, ABP increased the *B. longum* survival to *in vitro* gastrointestinal simulated conditions at 28 days of storage. The FSB supplemented with ABP showed to be a good vehicle for the probiotic tested, mainly for *B. longum* BB-46. Further studies, involving the potential health benefits of ABP are required.

Key-words: probiotic, acerola by-product, fermented soy beverage, probiotic survival, gastrointestinal simulation *in vitro*.

1. INTRODUCTION

Environmental issues along with the world's population growth and increased food production are big challenges for both industries and the governmental and non-governmental agencies of environmental protection. In fact, billions of tons of waste and by-products resulting from agriculture and food processing are generated every year, leading to serious problems, not only for the environmental, but also for the economy and food safety (Ravindran & Jaiswal, 2016, Lai et al., 2017, Mattsson, Williams, & Berghel, 2018). On the other hand, several reviews published point out to the fact that food processing by-products are excellent sources of complex carbohydrates like dietary fibres, proteins, polyunsaturated fatty acids and other lipids, as well as bioactive compounds such as antioxidant and other compounds with functional and nutraceutical proprieties, which may be employed in the development of innovative food, with a high aggregate value (Fava et al., 2013, Kowalska, Czajkowska, Cichowska, & Lenart, 2017, Lai et al., 2017, Mirabella, Castellani, & Sala, 2014).

In this context, studies have demonstrated that acerola (*Malpighia emarginata* DC) by-products are a good source of several bioactive compounds like dietary fibre, phenolic compounds, vitamin C, β-carotene, among other bioactive compounds (Duzzeioni, Lenton, Silva, & Barroso, 2013, Silva, Cazarin, Batista, & Maróstica Jr., 2014, Silva, Duarte, & Barrozo, 2016, Vieira, Bedani, Albuquerque, Biscola & Saad, 2017). In this line, Silva et al. (2014) compared the content of bioactive compounds in the pulp with that of the by-products of tropical fruits and observed that there was a higher content of anthocyanin and yellow flavonoids in the acerola by-product compared to its fruit pulp. In addition, Duzzeioni et al. (2013) highlighted that due to its high content of bioactive compounds, the acerola by-product may be used for several purposes, avoiding its disposal in the environment. Besides, studies using acerola by-product in association with probiotic cultures to evaluate its potential prebiotic effect have been conducted. In this context, Vieira et al., (2017) verified that acerola by-product can be selectively fermented by benefitial probiotic strains in an *in vitro* study. Albuquerque, Bedani, Vieira, LeBlanc, and Saad (2016) suggested that this by-product may also contribute for an increased folate production by lactobacilli and streptococci strains. Probiotics are defined as "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), and prebiotic is currently defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017).

In addition, a number of studies have demonstrated the application of vegetable by-products in the development of probiotic fermented products in both dairy and vegetable-based

products confers functional characteristics to these products. Espírito Santo et al. (2012), demonstrated that apple and banana by-products increased the probiotic viability during refrigerated storage of yoghurts. In another study, Espírito Santo et al. (2013) reported good sensory acceptability and increased viscosity of probiotic yoghurts supplemented with passion-fruit by-product. Sendra et al., (2008) reported that supplementation with citrus fibre increased the viability of probiotic bacteria in fermented milk. Albuquerque, Bedani, LeBlanc, & Saad (2017) reported that the addition of passion-fruit by-product and fructo-oligossacharide (FOS) in a fermented soymilk increased the production of folate by *Streptococcus thermophilus* strains alone or in co-culture with *Lactobacillus acidophilus* LA-5 and *L. rhamnosus* LGG. The increased firmness of soy yoghurt containing okara (a soybean by-product) was reported by Bedani, Campos, Castro, Rossi, and Saad (2013). In a complementary study, Bedani, Rossi, and Saad (2013) observed a high viability of probiotic strains (>8 log CFU/mL) in a soy "yoghurt" supplemented with okara and inulin, with a protective effect in the *Bifidobacterium animalis* BB-12 populations during the *in vitro* gastrointestinal (GI) simulation and an increased survival of *Lactobacillus acidophilus* LA-5 during the gastric phase in the soy yoghurts with the okara by-product.

The development of a fermented soy beverage (entirely of vegetal origin) with probiotic strains and acerola by-product powder (ABP) could result in an interesting new probiotic product, and also represent a promising alternative for the reduction of the disposal of fruit industrial by-products with high nutritional and biological properties (Duzzeioni et al., 2013, Silva et al., 2014, Silva et al., 2016, Vieira et al., 2017) and aggregated value. In a previous study, Vieira et al. (2017) showed that acerola by-product could be a potentially prebiotic ingredient. Therefore, the effect of ABP, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium longum* BB-46 on probiotic viability in a fermented soy beverage (FSB) and on probiotic survival under *in vitro* simulation GI conditions were investigated during 28 days of storage at 4 ºC.

2. MATERIAL AND METHODS

2.1. Microorganisms and acerola by-product obtain

The probiotic cultures *Lactobacillus acidophilus* LA-5 and *Bifidobacterium longum* BB-46 and the starter culture *Streptococcus thermophilus* TH-4 were supplied by Christian Hansen (Hørsholm, Denmark). The cultures employed in the fermented soy beverages were activated with two successive transfers from the stock cultures (-70 ºC) by inoculation in de Man, Rogosa & Sharpe broth (MRS broth, Oxoid, Basignstoke, UK) for LA-5; MRS broth

(Oxoid) containing L-cysteine hydrochloride hydrate (0.05%, w/v, Sigma-Aldrich, Germany) for BB-46; and in Hogg-Jago glucose broth (HJ) for TH-4, followed by incubation at 37 ºC in aerobic conditions for 24 h, except for BB-46, incubated under anaerobic condition (Anaerobic incubation system AnaeroGen®, Oxoid). After the second incubation, the inoculum was washed twice using a sterile NaCl solution, (0.85%, w/v) and the cells were collected by centrifugation at 8,600 *gx* for 10 min, and stored in an ice bath until the production of the fermented soy beverage. The acerola (*Malpighia emarginata* DC) by-product flour was obtained as a fine powder (<0.42 mm) as described by Vieira et al. (2017).

2.2. Experimental design and production of the fermented soy beverages.

A 2^3 factorial randomized design was employed for the fermented soy beverages (FSB) development. Eight pilot-scale-making formulations of FSB were produced in batches of 2.5 L, in triplicates (three randomized different batches of the same formulation, using a new water soluble soy extract and a new inoculum), in order to evaluate the addition of *Lactobacillus acidophilus* LA-5 (Chr. Hansen), as Factor 1 (X1), *Bifidobacterium longum* BB-46 (Chr. Hansen), as Factor 2 (X2), and the acerola by-product (ABP), as Factor 3 (X3) in two levels (presence or absence) during the production of the FSB (**Table 4.1**). The mixer Thermomix® (Vowerk) was used for the mixture and pasteurization of the soy base (**Table 4.2**) and the starter culture *Streptococcus thermophilus* TH-4 was inoculated in all fermented soy beverages to promote the pH decrease.

Table 4.1. Experimental design employed in the production of the fermented soy beverages.

Fermented soy beverages	Variables tested		
	***L. acidophilus* LA-5**	***B. longum* BB-46**	**Acerola by-product**
	X1	***X2***	***X3***
FSB1	-	-	-
FSB2	+	-	-
FSB3	-	+	-
FSB4	-	-	+
FSB5	+	+	-
FSB6	+	-	+
FSB7	-	+	+
FSB8	+	+	+

(+) = presence; (-) = absence

Table 4.2. Ingredients employed in the production of the fermented soy beverages.

Ingredients[1]	Products							
	FSB1	**FSB2**	**FSB3**	**FSB4**	**FSB5**	**FSB6**	**FSB7**	**FSB8**
Acerola by-product (ABP)	-	-	-	2.0	-	2.0	2.0	2.0
Soy extract powder	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sucrose	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Dextrose	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Carrageen gum	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Ingredient[2]	**Products**							
	FSB1	**FSB2**	**FSB3**	**FSB4**	**FSB5**	**FSB6**	**FSB7**	**FSB8**
Concentrated acerola juice	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

[1.]Ingredients in g/100 mL of water-soluble soy extract Mais Vita Pura Soja culinary use (Yoki®, Pouso Alegre, MG, Brazil); soy extract powder Mais Vita (Yoki®); Sucrose (Coopersucar-União, Limeira, SP, Brazil); Dextrose monohydrated ST (Roquette, France); Carrageen gum (Agargel Ind. e Com. Ltda, São Paulo, SP, Brazil); [2.] Ingredients in g/100 g of fermented soy-based, concentrate acerola juice (Acerola Jal, Citro-Nutri, Olaria, RJ, Brazil). See **Table 4.1** for the description of addition or not of *L. acidophilus* LA-5 and of *B. longum* BB-46 (Chr. Hansen).

The water-soluble soy extract was heated to 50 ºC under constant agitation to dissolve the sucrose and the dextrose and the heating continued until achieving 80 ºC, when the soy extract powder and the carrageen gum were added. Afterward, the mixture was pasteurized (5 min at 90 ºC). Before pasteurization, when the mixture achieved 90 ºC, the ABP was added in the formulations FSB4, FSB6, FSB7, and FSB8 (see **Tables 4.1** and **4.2**). After pasteurization, all soy bases were cooled in ice bath to 37 ºC, for the addition of the previously prepared inocula, and the fermentation took place at 37 ºC (B.O.D. incubator) until achieving pH 5.5. Next, the beverage was cooled and kept at 4 ºC for 16 h, when concentrated acerola juice (100 g/kg) was added and mixed. Then, the FSB was packaged in plastic containers and stored at 4 ºC for up to 28 days.

2.3. Enumetation of probiotic and starter cultures and pH assays

In order to determine the probiotic and starter viability in the fermented soy beverages after 1, 14, and 28 days of storage under refrigeration (4 ºC), 10 g portions of each fermented soy beverage samples were collected, mixed with 90 mL of saline sterile solution (0.85%, w/v) using a Bag Mixer 400 (Interscience, St. Nom, France) and aliquots of 3 mL of each dilution were homogenized with 17 mL of trisodium citrate dehydrate solution (2%, w/v) and incubated at 45 ºC for 30 min, followed centrifugation 8,600 *xg* for 10 min at 4 ºC. The resulting pellet was washed and resuspended in Tris EDTA (10 mM Tris-HCl, 1 mM EDTA, pH set 8) buffer, followed by freezing until the propidium monoazide (PMA) treatment and the DNA extraction

took place. The viability was determined employing quantitative Real-Time PCR (qPCR) combined with PMA treatment (PMA-qPCR), as will be described in item 2.5.1.

The pH values were determined in triplicate (three different containers of different batch, for each formulation at 1, 7, 14, 21, and 28 days of storage) performed in a pHmeter Orion, Three Stars model (Thermofisher Scientific, Waltham, MA, USA), equipped with a penetration electrode model 2A04 (Analyser, São Paulo, Brazil).

2.4. *In vitro* gastrointestinal simulation assay.

FSB samples were submitted to an *in vitro* gastrointestinal simulation in 3 steps (gastric, enteric I, and enteric II phases), carried out according to Buriti, Castro, and Saad (2010), with slight modification as follows. Aliquot of 30 mL of diluted FSB samples as described in item 2.3 were transferred to 6 sterile flasks, composing a total of 6 flasks contain the samples (3 dilutions carried out with 2 different samples of the same batch in the same storage period). Gastric enzymes (gastric pepsin and lipase with final concentration of 3 g/L and 0.9 mg/L, respectively, both from Sigma-Aldrich) were added to diluted samples of each FSB, the pH was adjusted with HCl 1 M to 2.2-2.5, and incubated in a metabolic water-bath (Dubnoff MA-095, Marconi, Piracicaba, Brazil) at 37 ºC for 2 h with constant agitation of approximate 150 rpm. After the gastric phase simulation, the pH of samples was adjusted to 5.0-5.5 using an alkaline solution pH 12 (NaOH [6 g/L] and NaH_2PO_4 [10.8 g/L], Synth). Bile (bovine bile, Merck) and pancreatine (pancreatine from porcine pancreas, Sigma-Adrich) was added to a final concentration of 10 g/L and of 1 g/L, respectively, and incubated again at 37 ºC for 2 h to simulate enteric phase I. Next, the pH was adjusted to 6.9-7.3 using the alkaline solution previously described, containing bile and pancreatine to maintain the concentration of 10 g/L and 1 g/L, respectively, and the samples were incubated again for 2 h at 37 ºC to simulate the enteric II phase, reaching 6 h of assay. To quantify the surviving populations of probiotic and starter cultures after the *in vitro* gastrointestinal simulations, the samples were processed as described in item 2.2.

2.5. Determination of probiotic and starter viability and survival under *in vitro* GI simulation using PMA-qPCR

To determine the probiotic and starter viability, as well as the survival under *in vitro* GI simulation, the samples were treated with PMA to amplify and quantify the DNA from living cells.

2.5.1 PMA-treatment and DNA extraction

The PMA treatment was carried out as described by Nocker, Cheung, and Camper (2006), with slight modifications described by Villarreal et al. (2013). The cell suspensions from viability and survival under *in vitro* GI simulation were centrifuged at 13,000 x*g* for 5 min at 4 ºC and resuspended with 500 µL of PBS buffer with PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride; Biotium, Inc., Hayward, CA, USA) to a final concentration of 50 mM. After the incubation in ice bath for 5 min in the dark, duplicate samples were light-exposed for 15 min at a distance of about 20 cm from a 650-W halogen light sources (DWE, 650 W, 120 V, GE, Lighting, East Cleveland, OH, USA) in the ice bath to prevent excessive temperature rise. After the PMA treatment, the samples were washed with PBS buffer, centrifuged (13,000 *xg* for 10 min at 4 ºC) and the cell pellet was resuspended in 500 µL of TE buffer prior to DNA isolation procedure. DNA extraction was carried according to Villarreal et al. (2013) and the resulting DNA was resuspended in 100 µL of TE buffer, and its quality and concentration were determined using a Nanodrop ND-1000 (Thermo Scientific, Waltham, USA).

2.5.2. Quantitative real-time PCR conditions

16S rRNA gene-targeted was used to enumerate the population of *Lactobacillus acidophilus* and the genomic DNA was used to enumerate the *Bifidobacterium longum* and *Streptococcus thermophilus* in the samples collected from assays for the enumeration of viablecell in the food product and *in vitro* GI survival. The reactions were performed using an ABI-PRISM 7500 sequencing detection system (Applied Biosystems, Bridge-water, NJ, USA). The reaction mixtures (25 µL) consisted of 12.5 µL of PCR Master Mix (Power SYBR® Green and TapMan® Universal Master Mix II, Life Technologies, ThermoFisher Scientific, USA), each primer at the appropriate concentration, 0.1 of bovine serum albumin (BSA, ThermoFisher) and 5 µL of the template DNA. For determination of *L. acidophilus*, *Bifidobacterium longum,* and *S. thermophilus*, primer concentration and amplification profiles program were described by Tabasco, Paarup, Janer, Peláez, and Reguena (2007), Furet et al. (2009), and Falentin et al. (2012), respectively. In order to quantify each target microorganisms, the standard curve was generated by a 10-fold serial dilution of genomic DNA and/or 16S rRNA gene (10^8-10^0 copies per µL) amplified from the respective target strains. For *L. acidophilus,* the 16S rRNA was used, considering the *L. acidophilus* NCFM strain as the genome's reference strain, with the number of copies of the16S rRNA gene in the *L. acidophilus* genome estimated as 4 (Altermann et al., 2005). Additionally, non-template controls (NTC) samples were carried

out in all qPCR runs as the negative control. The mean values from assays of viability and survival to in vitro simulated GI conditions were expressed in log CFU equivalent/mL.

2.6. Statistical analyses

The experiment constituted a 2^3 factorial design with three factors in two levels. Initially, data were verified regarding normality and homoscedasticity of variances, respectively, Shapiro-Wilk and Bartlett tests were used. Analysis of variance (ANOVA) was used for data for whicn normality and homoscedasticity were observed, and the Tukey's test was used for means comparison, in a 95% of confidence level. For data that not showing normality and homoscedasticity, the non-parametric Kruskal-Wallis test and the Fisher LSD test for mean comparison were used. The statistical package employed was Statistica 13.0 (StatSoft, Tulsa, OK, USA), the data were expressed as means ± standard deviation (SD) and the significant difference was established. Principal Components Analysis was performed to evaluate the relationships between the eight pilot-scale-marking formulation of the 2^3 factorial design (*L. acidophilus* LA-5, *B. longum* BB-46, and ABP) regarding the probiotic and starter cultures survival to simulated GI stress (scores of *t0*, *t2* and *t6*, for each microorganism) and the effect of the storage period (1, 14, and 28 days) independently. Covariance was the matrix type used in the mapping. The XLSTAT 2017 software was employed.

3. RESULTS AND DISCUSSION

3.1. pH values

The pH values of FSB during refrigerated storage are shown in **Table 4.3.** In general, the interaction of all study factors (LA-5*BB-46*ABP, see **Table 4.1**) was not significant ($P>0.05$) for the pH values. However, when each factor is evaluated independently, lower pH value was observed in the presence of *B. longum* BB-46 ($P<0.001$) and *L. acidophilus* ($P<0.05$). On the first day of storage, the FSB with *B. longum* (FSB3, FSB5, FSB7, and FSB8) presented the lowest pH values. On the other hand, higher pH values ($P<0.05$) were observed in the presence of ABP, except for FSB7 and FSB8. Additionally, a significant ($P<0.001$) decrease was verified in the pH values during the storage period for all formulations. Studies have shown that pH decreases in fermented products during storage were observed due to continuing metabolic activity of microorganisms, as a consequence of the population mass increases in the food fermented matrix (Costa, Soares Júnior, Rosa, Caliari, & Pimentel, 2017). Bedani et al. (2013) and Bedani, Vieira, Rossi, and Saad (2014) reported significant decreases

in pH values throughout refrigerated storage in synbiotic soy products similar to yoghurt. On the other hand, Battistini et al. (2018), using the same combination of probiotic and starter cultures (ABT-4, Chr. Hansen) in a soy-based fermented beverage, did not verify any significant difference in pH values between the first and the last day of storage. Additionally, some studies have demonstrated that the addition of fruit juices and pulps may reduce the pH values in soy products (Bedani et al., 2014; Granato, Branco, Nazzaro, Cruz, & Faria, 2010, İçier, Gündüz, Yilmaz, & Memeli, 2015) due to the low buffering effect of soy protein (Matias et al., 2016; Wang et al., 2009). Therefore, the continuous production of organic acids by the bacteria present in the product, the addition of concentrated acerola juice, and the low buffering ability of the soy proteins may have contributed for the pH decrease during storage in the present study.

3.2. Viability of probiotic and starter cultures

Propidium monoazide combined with real-time quantitative PCR (PMA-qPCR) was employed to evaluate the viability of *L. acidophilus* LA-5, *B. longum* BB-46, and *S. thermophilus* TH-4 obtained from the FSB formulations (**Table 4.4)**. There is still no consensus on the daily probiotic dose needed to promote health benefits. The probiotic dose-response is influenced by several factors, including desired health effect, probiotic strain, delivery matrix, and administration form. These variables make it difficult to generalise one optimal concentration to obtain a probiotic effect (Ouwehand, 2017). Nonetheless, Champagne, Ross, Saarela, Hansen, and Charalampopoulos (2011) pointed out that food must contain a minimum quantity of viable cells between 10^6 to 10^8 CFU/g or mL of food ready-to-eat. Additionally, the Canadian regulatory agency, the Health Canada, recommends that the ammount of viable cells of probiotic microorganisms in a product should provide a minimum daily dose of 10^7 CFU/day (Health Canada, 2015). Thus, all fermented soy beverages with added probiotic microorganisms (*L. acidophilus* and *B longum*) presented counts of viable cells above the minimum internationally recommended dose, during the shelf-life of the product, since during the whole refrigeration storage (4ºC), *S. thermophilus, L. acidophilus,* and *B. longum* counts were stable and ranged from 8.1 up to 9.4 log CFU equivalent/mL, 7.0 up to 7.7 log CFU equivalent/mL, and from 7.4 up to 8.2 log CFU equivalent/mL, respectively (**Table 4.4.**).

In general, *S. thermophilus* and *L. acidophilus* populations decreased significantly ($P<0.05$) during the refrigerated (4 ºC) storage. However, these changes during storage were only microbiologicaly significant for *S. thermophilus* in FSB3 and for *L. acidophilus* in FSB5 and FSB8. Similarly results were reported by Bedani, Rossi, and Saad (2013), who observed a

decrease in the viability of *L. acidophilus* in a soy yoghurt during storage, but which was of little microbiological significance, since these changes were always less than 0.5 log CFU/mL. In a later study, Bedani et al. (2014) verified a decrease in the LA-5 populations during storage of soy yoghurt added of guava and mango pulp, which was not observed in the control fermented soy yoghurt. The authors concluded that the addition of fruit pulps may reduce the viability of LA-5 during storage. In contrast with what was observed in the present study for *L. acidophilus* LA-5, Battistini et al., (2018) noted a higher decrease in *L. acidophilus* LA-5 up to 1.8 log CFU/mL in all fermented soy beverages, supplemented or not with inulin and FOS, but *B. animalis* and *S. thermophilus* populations did not change during the storage period. On the other hand, the populations of BB-46 of FSB3, FSB5, FSB7, and FSB8 showed no significant differences ($P>0.05$) in the storage period and/or for the different formulations, except at 14 days for the FSB8, which presented the lowest populations among all the formulations and among all sampling periods studied.

Table 4.3. Mean pH values of fermented soy products during refrigerated (4 ºC) storage.

Fermented soy beverage*	Time (days)				
	1	**7**	**14**	**21**	**28**
FSB1	5.05 (0.03)^{Ab}	5.01 (0.08)^{Abc}	4.99 (0.08)^{ABa}	4.85 (0.04)^{Cbc}	4.90 (0.07)^{BCa}
FSB2	4.98 (0.07)^{Ab}	4.93 (0.05)^{ABc}	4.94 (0.15)^{ABa}	4.83 (0.07)^{Cc}	4.86 (0.06)^{BCa}
FSB3	4.68 (0.06)^{Ac}	4.39 (0.09)^{Bd}	4.32 (0.06)^{BCb}	4.23 (0.06)^{CDe}	4.20 (0.04)^{Db}
FSB4	5.21 (0.04)^{Aa}	5.14 (0.08)^{ABa}	5.04 (0.04)^{Ca}	5.04 (0.11)^{BCa}	4.91 (0.15)^{Da}
FSB5	4.67 (0.13)^{Ac}	4.40 (0.02)^{Bd}	4.29 (0.10)^{Cb}	4.22 (0.05)^{Ce}	4.20 (0.08)^{Cb}
FSB6	5.13 (0.19)^{Aab}	5.07 (0.18)^{ABab}	4.97 (0.13)^{BCa}	4.94 (0.13)^{Cab}	4.83 (0.02)^{Da}
FSB7	4.49 (0.08)^{Ad}	4.41 (0.06)^{ABd}	4.28 (0.08)^{Cb}	4.32 (0.05)^{BCde}	4.23 (0.09)^{Cb}
FSB8	4.41 (0.01)^{Ad}	4.32 (0.03)^{ABd}	4.34 (0.11)^{ABb}	4.37 (0.10)^{ABd}	4.30 (0.08)^{Bb}

Values are expressed as mean (SD). $^{A-D}$ Different superscript capital letters in a row for each microorganism denote significant differences during storage period ($P<0.05$). $^{a-e}$ Different lowercase superscript letters in a column denote significant differences between fermented soy beverage formulation ($P<0.05$). *See **Table 4.2** for the fermented soy beverage description.

In general, *S. thermophilus* showed statistically and microbiologically lower populations ($P<0.05$) in the presence of *B. longum* and ABP (FSB8) on the first day of storage. In addition, reductions of microbiological significance in TH-4 populations were observed when this strain was in co-culture with BB-46, with a reduction of approximately 0.8 log CFU equivalent/mL for the FSB3 throughout the storage period. On the other hand, when TH-4 was in co-culture with LA-5, the TH-4 populations was not affected ($P<0.05$). Oliveira, Perego, Converti, and Oliveira (2009a) evaluated the effect of inulin in co-cultures of *Lactobacillus acidophilus*, *L. rhamnosus, L. bulgaricus*, and *B. lactis* with *Streptococcus thermophilus*, and

compared with their pure cultures. The authors did not verify any effects on the populations of probiotic strains or *S. thermophilus* in co-cultures. However, in a complementary study Oliveira, Perego, Converti, and Oliveira (2009b) evaluated the effect of inulin on kinetic parameters of growth and acidification of the same probiotic strains in co-culture with *S. thermophilus* and a pool composed of all tested strains. The authors observed higher populations of *L. rhamnosus* and *B. lactis* when these strains were in binary co-cultures with *S. thermophilus*, but in a pool with other probiotic cultures both strains presented lower populations ($P<0.05$) after 1 and 7 days of storage, due to a high competition for substrates.

The LA-5 and BB-46 populations were slightly lower in the presence of ABP, but no significant differences ($P<0.05$) were detected. In contrast, Zhao & Shah (2014) observed that different concentrations of tea extract added in a fermented soymilk decreased the viability of *B. longum* CSCC 5022 below > 4 log CFU/mL in the presence of green tea and black tea, four times and five times concentrated, respectively. However, this effect was not observed for *B. longum* CSCC 5089, since there was a reduction of less than 1 log CFU/mL for the higher tea concentrations.

Table 4.4. *Streptococcus thermophilus* TH-4, *Lactobacillus acidophilus* LA-5, and *Bifidobacterium* spp. (log CFU equivalent/mL) in fermented soy beverages during the refrigerated storage (4º C).

Fermented soy beverage*	Microorganisms								
	***Streptococcus thermophilus* TH-4**			***Lactobacillus acidophilus* LA-5**			***Bifidobacterium longum***		
	1 day	**14 days**	**28 days**	**1 day**	**14 days**	**28 days**	**1 day**	**14 days**	**28 days**
FSB1	9.33 (0.06)^{Aa}	9.30 (0.18)^{Aa}	9.38 (0.08)^{Aa}	-	-	-	-	-	-
FSB2	9.24 (0.04)^{Aa}	9.14 (0.36)^{Aab}	9.16 (0.11)^{Aab}	7.72 (0.04)^{Aa}	7.31 (0.33)^{Bab}	7.35 (0.56)^{Bab}	-	-	-
FSB3	9.06 (0.17)^{Aa}	8.64 (0.26)^{Bbc}	8.30 (0.43)^{Cc}	-	-	-	8.04 (0.38)^{Aa}	8.09 (0.31)^{Aa}	8.16 (0.24)^{Aa}
FSB4	9.11 (0.08)^{Aa}	8.90 (0.08)^{Ab}	8.98 (0.15)^{Ab}	-	-	-	-	-	-
FSB5	9.06 (0.23)^{Aa}	8.65 (0.11)^{Bbc}	8.84 (0.70)^{Ab}	7.66 (0.30)^{Aa}	7.43 (0.28)^{Aa}	6.99 (0.20)^{Bb}	7.84 (0.23)^{Aa}	7.84 (0.14)^{Aab}	7.97 (0.18)^{Aa}
FSB6	9.26 (0.14)^{Aa}	9.17 (0.33)^{Aab}	8.98 (0.19)^{Ab}	7.52 (0.18)^{Aa}	7.63 (0.07)^{Aa}	7.46 (0.18)^{Aa}	-	-	-
FSB7	8.72 (0.08)^{Ab}	8.51 (0.30)^{ABc}	8.30 (0.18)^{Bc}	-	-	-	7.69 (0.22)^{Aa}	8.00 (0.37)^{Aa}	7.98 (0.35)^{Aa}
FSB8	8.20 (0.03)^{Bc}	8.60 (0.27)^{Ac}	8.14 (0.10)^{Bc}	7.60 (0.18)^{Aa}	7.07 (0.43)^{Bb}	7.09 (0.09)^{Bb}	7.98 (0.23)^{Aa}	7.38 (0.35)^{Ab}	7.77 (0.53)^{Aa}

^{A,B} Different superscript capital letters in a row for each microorganism denote significant differences during storage period ($P<0.05$). ^{a,b,c} Different lowercase superscript letters in a column denote significant differences between fermented soy beverage formulation ($P<0.05$). - = not determined according to the experimental design (see **Table 4.1**). Values are expressed as mean (SD). *See **Tables 4.2** for fermented soy beverage description.

3.2. Survival of probiotic and starter microorganisms under *in vitro* simulated gastrointestinal conditions

The survival of *S. thermophilus, L. acidophilus*, and *B. longum* in the fermented soy beverages submitted to *in vitro* simulated GI conditions are shown in **Figures 4.1, 4.2** and **4.3**, respectively. The gastric phase (2 h) represented a limiting stage for the probiotic and *S. thermophilus* survival in the present study, since after 2 h of assay (gastric phase), populations of the three microorganisms studied showed reductions of up to 3.63 log CFU equivalent/mL for *S. thermophilus* TH-4 in FSB5 and 2.66 log CFU equivalent/mL for *B. longum* BB-46 in FSB7, both at 14 days of storage, and up to 5.89 log CFU equivalent/mL for *L. acidophilus* LA-5 in FSB6 at 28 days of storage. In general, a significant increase ($P<0.05$) in *L. acidophilus* and *S. thermophilus* survival was observed at 14 days of storage for the gastric phase. However, a significant decrease in the survival of both strains was observed after 28 days of storage for the gastric phase, while the *B. longum* populations decreased significantly ($P<0.05$) in all storage periods in the same phase. Bedani et al. (2013, 2014) demonstrated that highest reductions in the viable cell of *L. acidophilus* were observed in the gastric phase. Similar results were reported by Uriot et al. (2016) for some *S. thermophilus* strains since the higher decreases in the *S. thermophilus* populations were observed after 120 minutes of retention in the stomach compartment of the gastrointestinal dynamic *in vitro* model (TIM-1). Prasanna and Charalampopoulos (2018) showed that free cells with initial populations of *B. longum* subsp. *infantis* CCUG 52486 above 8 log CFU/mL was not detected after 120 minutes of the gastric treatment. Besides, at the first day of storage higher LA-5 population were observed in FSB8 comparing with the other formulations, which suggests that the co-culture with BB-46 and the presence of ABP may have contributed for this higher survival of LA-5. In addition, higher populations of BB-46 were observed in the formulations with LA-5 (FSB5), followed by the formulations which presented the probiotic co-culture and ABP (FSB8) in the same storage period and digestion phase. However, at 14 days fo storage, lower survival of LA-5 and BB-46 were observed, respectively, for FSB8 and FSB3 and at 28 days of storage, lower survival of probiotic strains was observed in FSB2 for LA-5 and in FSB7 for BB-46.

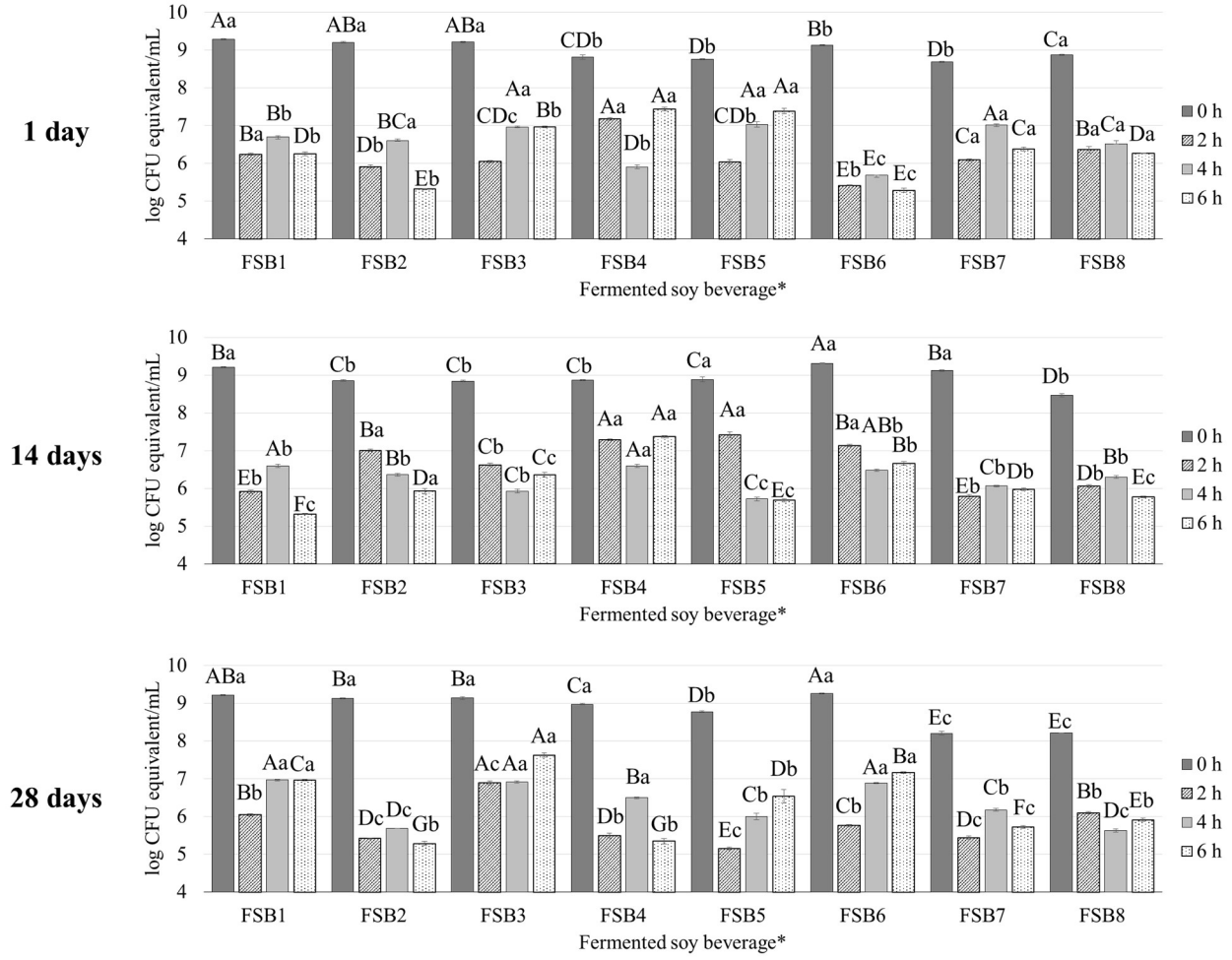


Figure 4.1. Survival of *S. thermophilus* TH-4 (log CFU equivalent/mL) in fermented soy beverages during storage for 1, 14, and 28 days, before (0 h) and during exposure to static *in vitro* simulated gastric (2 h) and enteric (4 h and 6 h) conditions. $^{A-F}$ Different superscript capital letters denote significant differences among fermented soy beverages for the same storage period and the same sampling period of the *in vitro* assay ($P<0.05$). $^{a-c}$ Different superscript lowercase letters denote significant differences between different sampling periods of the in vitro assay for the same formulation ($P<0.05$). *See **Table 4.2** for the description of fermented soy beverages.

Additionally, reductions in the populations of all evaluated microorganisms were observed between 0 h (before the assays) and 6 h (after enteric phase II) of the survival assay of up to 3.88 log CFU equivalent/mL, 5.26 log CFU equivalent/mL, and 1.95 log CFU equivalent/mL, respectively, for TH-4, LA-5, and BB-46. In the previous studies conducted by Bedani et al. (2013, 2014) with natural flavoured soy yoghurts and soy yoghurts containing fruit pulp, the authors observed a significant ($P<0.05$) decrease in the *L. acidophilus* LA-5 populations under GI conditions simulated *in vitro*. Similarly, Buriti et al. (2010) reported a higher decrease in the LA-5 survival during *in vitro* GI assay of mouses.

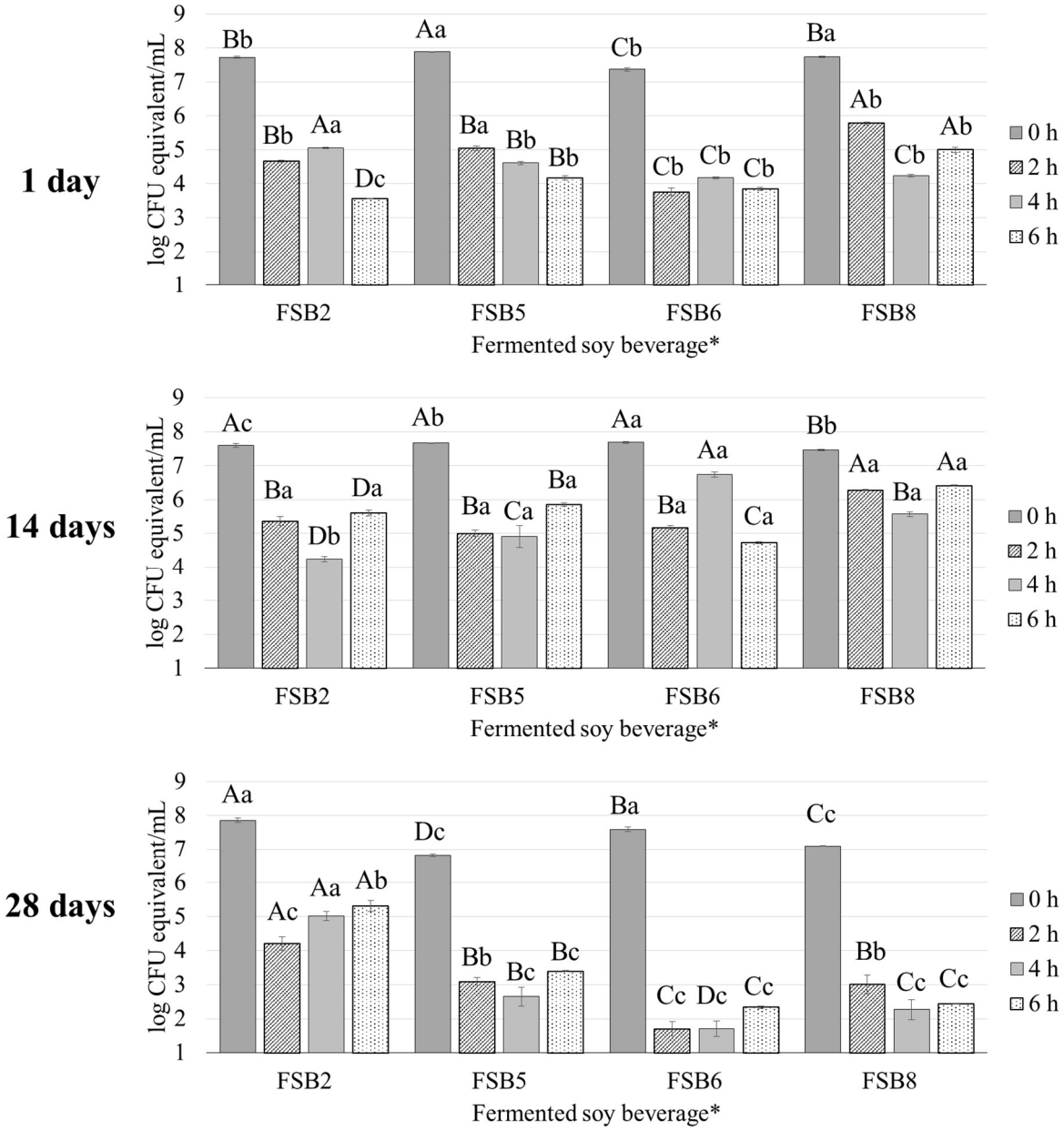


Figure 4.2. Survival of *L. acidophilus* LA-5 (log CFU equivalent/mL) in fermented soy beverages during storage for 1, 14, and 28 days, before (0 h) and during exposure to static *in vitro* simulated gastric (2 h) and enteric (4 h and 6 h) conditions. $^{A-D}$ Different superscript capital letters denote significant differences among fermented soy beverages for the same storage period and the same sampling period of the *in vitro* assay ($P<0.05$). $^{a-c}$ Different superscript lowercase letters denote significant differences between different sampling periods of the in vitro assay for the same formulation ($P<0.05$). *See **Table 4.2** for description of fermented soy beverages.

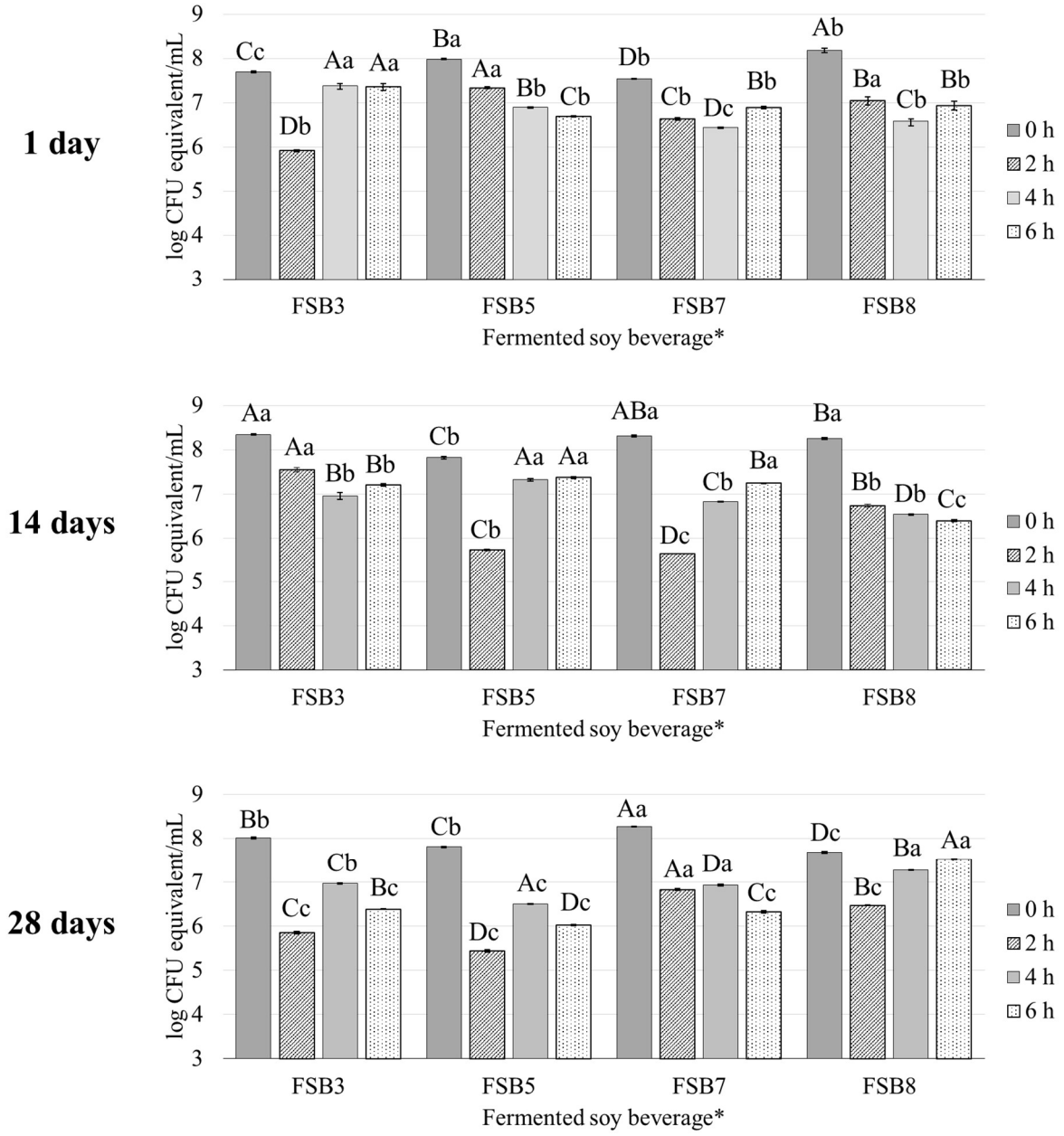


Figure 4.3. Survival of *Bifidobacterium longum* BB-46 (log CFU equivalent/mL) in fermented soy beverages during storage for 1, 14, and 28 days, before (0 h) and during exposure to static *in vitro* simulated gastric (2 h) and enteric (4 h and 6 h) conditions. $^{A-D}$ Different superscript capital letters denote significant differences among fermented soy beverages for the same storage period and the same sampling period of the *in vitro* assay ($P<0.05$). $^{a-c}$ Different superscript lowercase letters denote significant differences between different sampling periods of the in vitro assay for the same formulation ($P<0.05$). *See **Table 4.2** for the description of fermented soy beverages.

Similarly to what was observed in the gastric phase, there was an increase in the survival at 14 days of storage and a decrease at 28 days for *L. acidophilus* and *S. thermophilus*, as well as a decrease in the *B. longum* population for all storage periods in the enteric phase II (after 6 h of the assay). In addition, the presence of acerola by-product influenced the survival of strains in the gastric phase in different forms. The presence of ABP increased significantly ($P<0.05$) the *B. longum* survival after 28 days of storage. In this sense, Matias et al. (2016)

showed that different formulations of ice cream influenced the survival of *L. acidophilus* and *B. animalis* significantly after *in vitro* simulated GI stress assay. Buriti et al. (2010) reported that milk fat, inulin, and whey protein concentrated (WPC) present in the different formulations of mousses exposed to refrigerated and frozen storage influenced the survival of *L. acidophilus* in different ways and reported an increased probiotic survival in the presence of inulin in refrigerated mousses.

As described above by Buriti et al. (2010) and Matias et al. (2016), the formulations of probiotic and synbiotic products have a significant influence in the microorganism survival under *in vitro* GI simulated conditions. Therefore, to evaluate which formulation of the 2^3 factorial design presented important differences regarding the survival of *L. acidophilus, B. longum*, and *S. thermophilus* after the gastric and enteric phases of the *in vitro* GI simulation, we carried out a principal component analysis (PCA) on a dataset of the surviving population of each microorganism for each *in vitro* simulated GI phase (before assay – *0 h*, gastric phase – *2 h*, and enteric phase II – *6 h*). The refrigeration storage period (1, 14, and 28 days) also was considered in a second analyse. **Figure 4.4** shows the effect of each fermented soy formulation on the surviving populations of *Bifidobacterium longum* (4.4A), *Lactobacillus acidophilus* (4.4B), and *S. thermophilus* (4.4C) under *in vitro* simulated GI condition of the different fermented soy beverages, while **Figure 4.5** shows the effect on the surviving population of *B. longum* (**Figure 4.5A**), *L. acidophilus* (**Figure 4.5B**), and *S. thermophilus* (**Figure 4.5C**) under *in vitro* simulated GI condition of the different fermented soy formulations, for the different refrigeration storage periods (1, 14, and 28 days at 4 ºC).

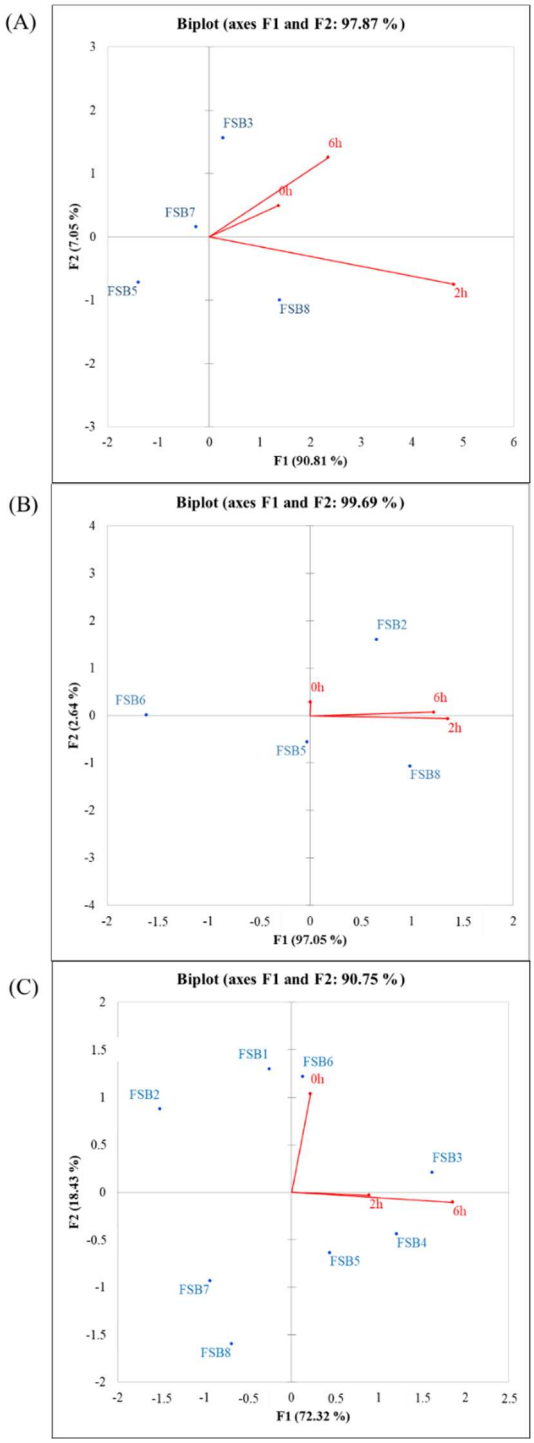


Figure 4.4. Principal Component Analysis (PCA) graphs of general survival of *Bifidobacterium longum* BB-46 (A), *Lactobacillus acidophilus* (B) and *Streptococcus thermophilus* TH-4 (C) in the fermented soy beverages (blue), before (0 h) and during exposure to static *in vitro* simulated gastric (2 h) and enteric (6 h) conditions (red). *See **Table 4.2** for the description of fermented soy beverages.

The first two principal components explained 98% of variability among different fermented soy beverages in relation to *B. longum* survivor (Figure 4.4A). Before the *in vitro* GI simulation (0h), the difference among *B. longum* populations in FSB3, FSB5, FSB7, and FSB8 (see **Table 4.2** to description of FSBs) was lower, according to the short vector represented (**Figure 4.4A**). The fermented soy beverage FSB8 presented the highest survival of *B. longum* compared to the other FSB in the gastric phase (2 h), while the FSB5 presented the lowest populations after the end of the enteric phase (6 h) and FSB3 had the highest survival in the same phase. When we include the effect of the storage period in the *B. longum* survival, the two first PC explain together 92.5% of the variation among the different fermented soy beverages (**Figure 4.5A**). As was observed for the formulation effect on the survival, the storage period showed a lower influence in the initial *B. longum* populations (0 h). Additionally, it was noted that the last storage period (28 days) tended to have a lower survival proportionally to the other storage periods in the enteric phase II, except for FSB8, which presented a higher survival of *B. longum* in this phase at 28 days of storage. Besides, for the gastric phase (2 h), it was observed that on the first day of storage FSB5 and FSB7 showed higher populations than FSB3 in the same storage period. On the other hand, in this same phase, FSB5 and FSB7 presented a lower survival than FSB8 and, mainly, FSB3 at 14 days of storage.

The two first PC for the effect of different FSB under *L. acidophilus* was able to explain 99.69% of the variation (**Figure 4.4B**), and when we also include the effect of the storage period, it is able to explain 97.84% of the variation (**Figure 4.5B**). In both PC graphics (**Figures 4.4B** and **4.5B**), the differences between the initial populations of *L. acidophilus* (0 h) and *B. longum*, was much lower for the fermented soy beverages and proportional in all storage periods. FSB6 showed the lowest survival after the gastric phase and the enteric phase II, compared to the other formulations with *L. acidophilus*. In the last storage period (28 days) it was verified that the fermented soy beverage tended to have a lower survival proportionally to the other gastric phase (2 h) and the enteric phase (6 h), except for FSB2 (with only *L. acidophilus*), which presented an average survival on this last day, mainly in the final phase (6h). The highest population of *L. acidophilus* after 6 h of trial tended to be on 14 days of storage for all fermented soy beverages, being slightly lower for FSB6. The same was observed after 2 h of the trial (gastric phase) for the same FSB, though, FSB8 and FSB5 also presented populations more similar to the other FSBs.

The effect of different FSBs on *S. thermophilus* survival after GI simulation was shown in **Figure 4.4C**. The first two principal component explained 90.75% of the variability. The size of the vector for the end of the simulated GI assay (6 h) was higher for the different FSB

than after the gastric phase and the initial populations. The lower initial populations (0 h) of *S. thermophilus* were observed in FSB7 and FSB8 in comparison with the other FSB, mainly FSB1 (absence of probiotic and ABP) and FSB6 (presence of LA-5 and ABP) that presented the highest initial populations (0 h). After the gastric phase and mainly after the end of the enteric phase, FSB3 and FSB4 presented higher populations than other FSB. Lower survival was verified at 6h of assay for FSB2, followed by FSB7 and FSB8. On the other hand, the two first PC for the storage effect under *S. thermophilus* survival for the different FSB explain 91.3% of the variation (**Figure 4.5C**), and the FSBs differences in the initial populations (0 h) was lower than the other simulated GI assay phase. In the first day of storage, *S. thermophilus* populations in FSB2 and FSB6 was lower in both gastric and enteric phases. The higher populations of *S. thermophilus* was observed for FSB4 in both phases and also for FSB3 and FSB5 after 6 h of GIT trial. On the 14th day of storage, the fermented soy beverages FSB2, FSB4, FSB5, and FSB6 showed higher survival of *S. thermophilus* than the other FSB, for the same storage day, after the gastric phase. On the 28th day of storage, FSB1, FSB3, and FSB6 tended to have a higher survival of *S. thermophilus* than the other FSB after the end of the enteric phase. In the gastric phase, FSB5 was highlighted by the lower survival to 28 days of storage.

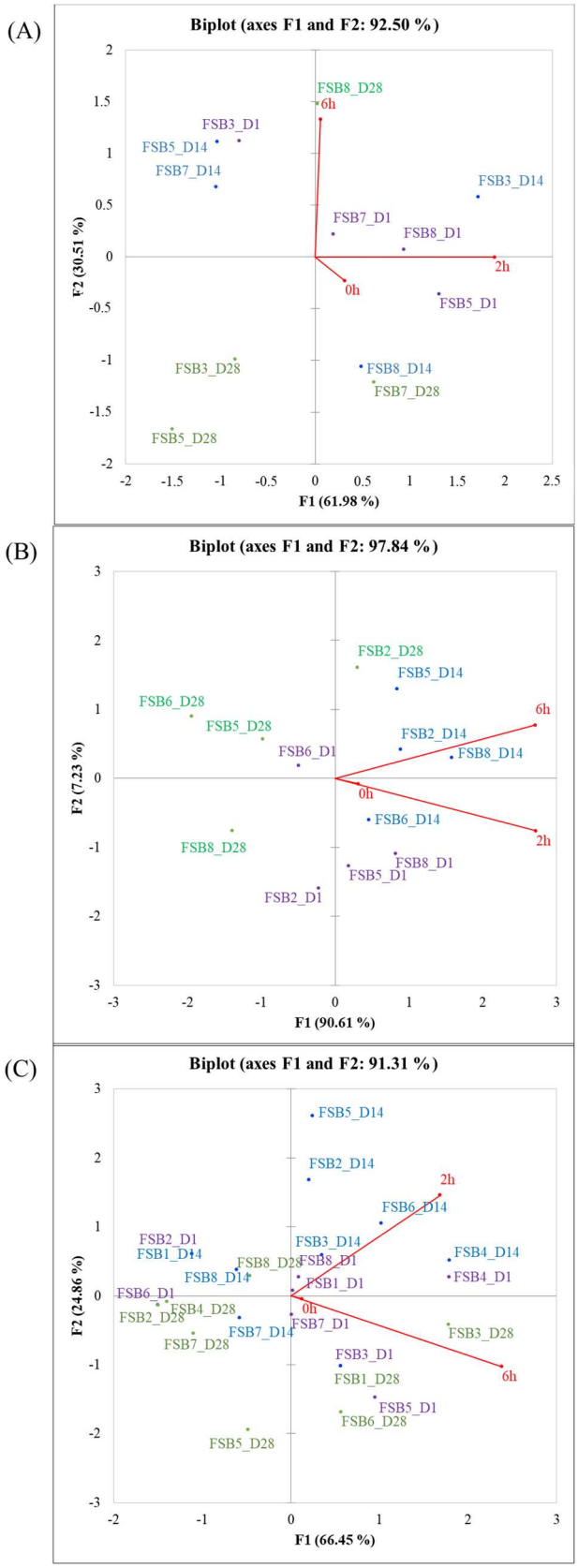


Figure 4.5. Principal Component Analysis (PCA) graphs of survival of *Bifidobacterium longum* BB-46 (A), *Lactobacillus acidophilus* (B) and *Streptococcus thermophilus* TH-4 (C) in the fermented soy beverages during storage of 1 (purple), 14 (blue), and 28 (green) days, before (0 h) and during exposure to static *in vitro* simulated gastric (2 h) and enteric (6 h) conditions (red). *See **Table 4.2** for the description of fermented soy beverages.

4. CONCLUSION

High viabilities of probiotics and starter cultures were observed for all the FSB, from 7 to 8 log CFU equivalent/mL and above 9 log CFU equivalent/mL, respectively, during the 28 days of shelf-life. The presence of ABP and of the co-cultures did not affect the viability of both probiotic microorganisms and of *S. thermophilus*. Reductions in the survival of probiotic and starter cultures were observed after the end of the GI *in vitro* simulations (6 h), during the different storage periods evaluated. In general, the lowest populations of probiotic and starter culture were observed at 28 days of storage, when compared to days 1 and 14 of storage, with a clear exception of *B. longum* BB-46, which showed the highest survival at 28 days of storage in FSB8, containing the probiotic combination and ABP. *L. acidophilus* LA-5 showed the highest surviving populations at 14 days of storage. The highest surviving populations of *S. thermophilus* TH-4 were observed during the first 14 days of storage in FSB4, containing ABP, but without the probiotic combination, with a higher decrease of the surviving populations at 28 days of storage for this beverage. Even though reductions in the survival of all microorganisms were observed at 28 days of storage, the FSB matrix with acerola by-product may be considered as a good vehicle for the probiotic strains tested against simulated gastrointestinal conditions, mainly for *B. longum* BB-46, which showed the lowest reductions in its population during the GI conditions simulated *in vitro*. Further studies involving the potential health benefits of acerola by-product incorporated in the fermented soy beverage containing probiotic strains like *B. longum* BB-46 are required.

5. REFERENCES

Albuquerque, M.A.C., Bedani, R., LeBlanc, J.G., & Saad, S.M.I. (2017). Passion fruit by-product and fructooligosaccharides stimulate the growth of folate production by starter and probiotic cultures in fermented soymilk. *International Journal of Food Microbiology, 261*, 35-41.

Albuquerque, M.A.C., Bedani, R., Vieira, A.D.S., LeBlanc, J. G., & Saad, S.M.I. (2016). Supplementation with fruit and okara soy by-products and amaranth flour increases the folate production by starter and probiotic culture. *International Journal of Food Microbiology*, *236*, 26-32.

Altermann, E., Russell, W.M., Azacarate-Peril, M.A., Barrangou, R., Buck, B.L., McAuliffe, O., et al. (2005). Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *PNAS, 102(11),* 3906-3912.

Battistini, C., Gullón, B., Ichimura, R.S., Gomes, A.M.P., Ribeiro, E.P., Kunigk, L., et al. (2018) Development and characterization of an innovative synbiotic fermented beverage based on vegetable soybean. *Brazilian Journal of Microbiology*, 49(2):303-309

Bedani, R., Campos, M.M.S, Castro, I.A., Rossi, E.A., & Saad, S.M.I. (2013). Incorporation of soy by-product okara and inulin in a probiotic soy yoghurt: texture profile and sensory acceptance. *Journal of the Science of Food and Agriculture*, *94*, 119-125.

Bedani, R., Rossi, E.A., & Saad, S.M.I. (2013). Impact of inulin and okara on *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under *in vitro* simulated gastrointestinal conditions. *Food Microbiology*, *34*, 382-389.

Bedani, R., Rossi, E.A., Cavallini, D.C.U., Pinto, R.A., Vendramini, R.C., Augursto, E.M., et al. (2015). Influence of daily consumption of synbiotic soy-based product supplemented with okara soybean by-product on risk factors for cardiovascular diseases. *Food Research International*, *73*, 142-148.

Bedani, R., Vieira, A.D.S., Rossi, E.A., & Saad, S.M.I. (2014). Tropical fruit pulp decreased probiotic survival to in vitro gastrointestinal stress in synbiotic soy yoghurt with okara during storage. *LWT – Food Science and Technology*, *55*, 436-443.

Buriti, F.C.A., Castro, I.A., & Saad, S.M.I. (2010). Viability of *Lactobacillus acidophilus* in symbiotic guava mousses and its survival under *in vitro* simulated gastrointestinal conditions. *International Journal of Food Microbiology, 137*, 121-129.

Champagne, C.P., Ross, R.P., Saarela, M., Hansen, K.F., & Charalampopoulos, D. (2011). Recommendation for the viability assessment of probiotics as concentrated cultures and in food matrices. *International Journal of Food Microbiology, 149,* 185-195.

Costa, K.K.F.D., Soares Júnior, M.S., Rosa, S.I.R., Caliari, M., & Pimentel, T.C. (2017). Changes of probiotic fermented drink obtained from soy and rice by-products during cold storage. *LWT-Food Science and Technology, 78*, 23-30.

Duzzioni, A.G., Lenton, V.M., Silva, D.I.S., & Barrozo, M.A.S. (2013). Effect of drying kinetics on main bioactive compounds and antioxidant activity of acerola (*Malpighia emarginata* D.C.) residue. *International Journal of Food Science and Technology*, *48*, 1041-1047.

El-Gawad, I.A.A., El-Sayed, E.M., Hafez, S.A., El-Zeini, H.M. & Saleh, F.A. (2005). The hypocholesterolaemic effect of milk yoghurt and soy-yoghurt containing bifidobacteria in rats fed on a cholesterol-enriched diet. *International Dairy Journal, 15*, 37-44.

Espírito Santo, A.P., Cartolano, N.S., Silva, T.F., Soares, F.A.S.M., Gioielli, L.A., Perego, P., et al. (2012). Fiber from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. *International Journal of Food Microbiology*, *154*, 135-144.

Espírito-Santo, A.P., Lagazzo, A., Sousa, A.L.O.P., Perego, P., Converti, A., & Oliveira, M.N. (2013). Rheology, spontaneous whey separation, microstructure and sensorial characteristics of probiotic yoghurts enriched with passion fruit fiber. *Food Research International, 50,* 224-231.

Falentin, H., Henaff, N., Le Bivic, P., Deutsch, S.-M., Parayre, S., Richoux, R., et al. (2012). Reverse transcription quantitative PCR revealed persistency of thermophilic lactic acid bacteria metabolic activity until the end of the ripening of Emmental cheese. *Food Microbiology, 29,* 132-140.

Fava, F., Zanaroli, G., Vannini, L., Guerzoni, E., Bordoni, A., Viaggi, D. et al. (2013). New advances in the integrated manafement of food processing by-products in Europe: sustainable exploitation of fruit and cereal processing by-products with the production of new food products (NAMASTE EU). *New Biotechnology, 30(6),* 647-655.

Furet, J.-P., Firmesse, O.; Gourmelon, M. Bridonneau, C.; Tap, J.; Mondot, S. et al. (2009). Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. *FEMS*

Microbial Ecology, 68, 351-362.

Gibson, G.R., Hutkins, R., Sanders, M.E., Prescott, S.L., Reimer, R.A., Salminen, S.J. et al. (2017). The Ineternatinal Scientific Association for Probiotic and Prebiotics (ISAPP) consensus statement on the definition ans scope of prebiotics. *Narute Reviews Gastroenterology & Hepatology, 14(8)*, 491-502.

Granato, D., Branco, G.F., Nazzaro, F., Cruz, A.G., & Faria, A.F. (2010). Functional foods and nondairy probiotic food development: Trends concepts, and products. *Comprehensive Reviews in Food Science and Food Safety, 9(3)*, 292-302.

Health Canada (2015). Natural health product: probiotics. http://webprod.hc-sc.gc.ca/nhpid-bdipsn/atReq.do?atid=probio Accessed 20 Dezember 2017.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B. et al. (2014). The International Scientific Association for Probiotic and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology, 11,* 506-514.

İçier, F., Gündüz, G.T., Yilmaz, B., & Memeli, Z. (2015). Changes on some quality characteristics of fermented soy milk beverage with added apple juice. *LWT–Food Science and Technology, 63*, 57-64.

Kowalska, H., Czajkowska, K., Cichowska, J., & Lenart, A. (2017). What's new in biopotential of fruit and vegetable by-products applied in the food processing industry? *Trends in Food Science & Technology,* 67, 150-159.

Lai, W.T., Khong, N.M.H., Lim, S.S., Hee, Y.Y., Sim, B.I., Lau, K.Y., et al. (2017). A review: Modified agricultural by-products for the development and fortification of food products and nutraceuticals. *Trends in Food Science & Technology, 59,*148-160.

Matias, N.S., Padilha, M., Bedani, R., & Saad, S.M.I. (2016). *In vitro* gastrointestinal resistance of *Lactobacllus acidophilus* LA-5 and *Bifidobacterium animalis* Bb-12 in soy and/or milk-base synbiotic apple ice creams. *International Journal of Food Microbiology, 234*, 83-93.

Mattsson, L., Williams, H., & Berghel, J. (2018). Waste of fresh fruit and vegetables at retailers in Sweden – Measuring and calculation of mass, economic cost and climate impact. *Resources, Conservation & Recycling, 130*, 118-126.

Mirabella, N., Castellani, V., & Sala, S. (2014). Current options for the valorization of food manufacturing waste: a review. *Journal of Cleaner Production, 65*, 28-41.

Nocker, A., Cheung, C.-Y., & Camper, A.K. (2006). Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods, 67*, 310-320.

Oliveira, R.P.S., Perego, P., Converti, A., & Oliveira, M.N. (2009a). Growth and acidification performance of probiotics in pure culture and co-cultures with *Streptococcus thermophilus*: The effect of inulin. *LWT-Food Science and Technology*, *42,* 1015-1021.

Oliveira, R.P.S., Perego, P., Converti, A., & Oliveira, M.N. (2009b). Effect of inulin on growth and acidification performance of differente probiotic bacteria in co-culture and mixed culture with *Streptococcus thermophilus*. *Journal of Food Engineering, 91*, 133-139.

Ouwehand, A.C. (2017). A review of dose-responses of probiotic in human studies. *Beneficial Microbes, 8(2)*, 143-151.

Prasanna, P.H.P., & Charalampopoulos, D. (2018). Encapsulation of *Bifidobacterium longum* in

alginate-dairy matrices and survival in simulated gastrointestinal conditions, refrigeration, cow milk and goat milk. *Food Bioscience, 21*, 72-79.

Ravindran, R. & Jaiswal, A.K. (2016). Exploitation of food industry waste for high-value products. *Trends in Biotechnology, 34(1),* 58-69.

Sendra, E., Fayos, P., Lario, Y., Fernández-López, J., Sayas-Barberá, E., & Pérez-Alvarez, J.A. (2008). Incorporation of citrus fibers in fermented milk containing probiotic bacteria. *Food Microbiology, 25*, 13-21.

Silva, J.K., Cazarin, C.B.B., Batista, A.G., & Maróstica Jr., M. (2014). Effect of passion-fruit (*Passiflora edulis*) byproduct intake in antioxidant status of Wistar rats tissues. *LWT – Food Science and Technology*, *59*, 1213-1219.

Silva, P.B., Duarte, C.R., & Barrozo, M.A.S. (2016). Dehydration of acerola (Malpighia emarginata D.C.) residue in a new designed rotary dryer: Effect of process variables on main bioactive compounds. *Food and Bioproducts processing, 98*, 62-70.

Singh, B.P., & Vij, S. (2017). Growth and bioactive peptides production potential of *Lactobacillus plantarum* strain C2 in soy milk: A LC-MS/MS based revelation for peptides biofunctionality. LWT-*Food Science and Technology*, *86*, 293-301.

Tabasco, R., Paarup, T., Janer, C., Peláez, C., & Requena, T. (2007). Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, L. acidophilus, L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermentes milk. *International Dairy Journal, 17*, 1107-1114.

Uriot, O., Galia, W., Awussi, A.A., Perrin, C., Denis, S., Chalancon, S. et al. (2016). Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human but. *Food Microbiology, 53*, 18-29.

Vieira, A.D.S., Bedani, R., Albuquerque, M.A.C., Biscola, V., & Saad, S.M.I. (2017). The impact of fruit and soybean by-products and amaranth on the growth of probiotic and starter microorganisms. *Food Research International, 97*, 356-363.

Villarreal, M.L.M., Padilha, M., Vieira, A.D.S., Franco, B.D.G.M., Martinez, R.C.R. & Saad, S.M.I. (2013). Advantageous direct quantification of viable closely related probiotic in *petit-suisse* cheeses under *in vitro* gastrointestinal conditions by propidium monoazide-qPCR. *PlosOne, 8(12),* e82102.

Wang, J., Guo, Z., Zhang, Q., Yan, L., Chen, W., Liu, X.-M. et al. (2009). Fermentation characteristics and transit tolerance of probiotic *Lactobacillus casei* Zhang in soymilk and bovine milk during storage. *Journal of Dairy Science, 92*, 2468-2476.

Xavier-Santos, D., Lima, E.D., Simão, A.N.C., Bedani, R., & Saad, S.M.I. (2018) Effect of the consumption of a synbiotic diet mousse containg *Lactobacillus acidophilus* LA-5 by individuals with metabolic syndrome: A randomized controlled trial. *Journal of Functional Foods, 41*, 55-61.

Yeo, S.-K., & Liong, M.-T. (2010). Angiotensin I-converting enzyme inhibitory activity and bioconversion of isoflavones by probiotics in soymilk supplemented with prebiotics. *International Journal of Food Science and Nutrition, 61(2),* 161-181.

Zhao, D. & Shah, N.P. (2014). Influence of tea extract supplementation on bifidobacteria during soymilk fermentation. *International Journal of Food Microbiology, 188*, 36-44.

Chapter 5

Effect of supplementation of a probiotic soy beverage with acerola by-product (Malpighia emarginata DC) on microbial modulation of Dutch lean and obese individuals

Manuscript to be submitted for publication as:

VIEIRA, A.D.S.; SOUZA, C.B.; TENAS-CUEVAS, M.; HOFFMANN, C.; ZOETENDAL, E.G.; SMIDT, H.; SAAD, S.M.I.; VENEMA, K. *Effect of supplementation of a probiotic soy beverage with acerola by-product (Malpighia emarginata DC) on microbial modulation of Dutch lean and obese individuals.*

ABSTRACT

Background: Obesity and other diseases have been associated with a reduction in the bacterial diversity of the gut microbiota. Additionally, recent studies demonstrate that the use of fruit by-products with a high content of bioactive compounds may be associated with different effects on the host's health, as it seems to increase the diversity of the gut microbiota and reduce the risk for the onset of some diseases. Therefore, the effect of a fermented soy beverage with probiotic strains and acerola by-product on the modulation of composition and activity of lean and obese microbiota using the intestinal *in vitro* model TIM-2 was evaluated.

Result: The acetate and lactate production by the lean microbiota for fermented soy beverage meals (SF1–without probiotic or acerola by-product (ABP); SF2–with *Lactobacillus acidophilus* LA-5 + *Bifidobacterium longum* BB-46, but without ABP; SF3–without probiotics, but with ABP; and SF4–with both LA-5+BB-46 and ABP) were much higher than that produced by the obese microbiota and/or for the control meal for both microbiotas, leading to a significantly higher ($P<0.05$) total SCFA content and energy extraction for the lean microbiota. In addition, lactate production increased significantly ($P<0.05$) for the meals containing probiotics combined with ABP than control, while the acetate increased significantly ($P<0.05$) in the presence of probiotics without ABP, also than control. The unweighted PCoA showed a clear separation between the lean and the obese microbiota, while the weighted PCoA showed that after 48 h of fermentation, the obese microbiota moved into the space occupied by the lean microbiota. *Bifidobacterium* spp. populations were higher in the lean microbiota and *Lactobacillus* spp. populations were higher in the obese microbiota. Additionally, high counts of *B. longum* were observed in both the lean and the obese microbiota, with a mean ratio between the *B. longum/Bifidobacterium* spp. of up to 64% in SF1 at 24 h for the lean microbiota and to 99% in SF3 at 48 h for the obese microbiota. On the other hand, *L. acidophilus* populations were lower in both the lean and the obese microbiota, presenting mean ratios between *L. acidophilus/Lactobacillus* spp. below 0.2%.

Conclusion: Fermented soy beverage supplemented with ABP presented the best characteristic regarding modulation of the obese microbiota, with an increase in beneficial bacteria from the genera *Bifidobacterium* and *Lactobacillus.* Moreover, after 48 hours of intervention the obese microbiota was apparently similar to the lean microbiota.

Key-words: Probiotic, acerola by-product, fermented soy beverage, intestinal in vitro model, human microbiota modulation

1. BACKGROUND

The World Health Organization (WHO) estimated that, in 2014, more than 1.9 billion adults aged 18 years and older were overweight, of these over 600 million adults were obese. In general, about 13% of the world's adult population were obese and 39% were overweight, in 2014. Additionally, in the same year, it was estimated that 41 million children under 5 years of age were overweight or obese. The obesity worldwide prevalence more than doubled between 1980 and 2014 [1]. At first considered a developed countries problem, overweight and obesity are now on the rise in low- and middle-income countries, particularly in urban settings. The impact of dietary habit and lifestyle is currently under investigation, but the role of other predisposing factors, such as genetic determinants and family history, still needs to be elucidated. In humans, obese subjects are supposed to have a more efficient microbiota in energy extraction from food, due to the detection of quantitative difference in the major bacterial group in obese subjects compared to lean ones [2].

Nowadays, the studies are intensifying to find out the causative factors of obesity, due the high worldwide worry about the serious pathological role of obesity. Gut microbiota has gained a growing interest as an environmental factor that may affect the predisposition towards adiposity [3]. Recent discoveries in this field have shown a possible relationship between the composition patterns of the intestinal microbiota and obesity [4]. Studies have evidenced that a high-fat diet may induce changes in the gut microbiota in animal models regardless of the presence of obesity. In humans, obesity and other disorders as type 2 diabetes mellitus (T2D) have been associated with reduced bacterial diversity and an altered representation of bacterial species, but the identified differences are not homogeneous among the studies [4,5].

The human gut works is the home to a vast number of microorganisms, the microbiota, and their genomes complement our own set of genes. The gut microbiota functions at the intersection between host genotype and diet to modulate host physiology and metabolism, and recent data revealed that the gut microbiota could affect obesity [6]. The gut microbiota contributes to host metabolism by several mechanisms including increased energy harvest from diet, modulation of lipid metabolism, altered endocrine function, and increased inflammatory tone. The gut microbiota could thus be considered to be an environmental factor that modulates obesity and other metabolic disease [6,7]. The diet composition presents a crucial factor in the control of the communities of microorganisms of the gut microbiota. The quality and amount of macronutrients as carbohydrates, proteins, and fats from the diet present a high impact on the intestinal microbiota and, thus in the prevention and treatment of some diseases [8,9]. In a review, intestinal microbiota dysbiosis were reported by Jonkers [10] for subjects with any food

or nutritional disturbance, whether due to poor diet and/or malabsorption. In view of this, research has been conducted and demonstrated that specific modification of the diet can modulated the gut microbiota by reducing the production of undesirables metabolites, while increasing the beneficial microorganisms and metabolites, thus preventing and treating diseases associated with obesity. Intestinal microbiota modulation by probiotics and prebiotics, alone or in combination (synbiotics) have been pointed as a promising alternative to prevent and treatment of obesity and related disease [11]. Zhang et al. [6] demonstrated that the manipulation of diet, including the nondigestibles carbohydrates (amongst which prebiotics), of morbidly obese children modified the gut microbiota for a more healthy composition, with a decrease of bacteria that can produce potentially toxic metabolites and an increase of level of bacteria that produced potential beneficial metabolites. Additionally, Bagarolli et al. [12] described that probiotic strains modulated the gut microbiota in an *in vivo* model using diet-induced obesity mice (DIO mice), reversing the obesity-related characteristics, inducing an increase in hypothalamic insulin resistance. Rasmussen and Hamaker [13] showed that prebiotics and dietary fibres, when fermented by gut microbiota, resulted in the modulation of this intestinal microbiota and in the production of metabolic compounds with beneficial effect such as short-chain fatty acids (SCFA), which could reduce the inflammation process in inflammatory bowel disease.

The identification of bioactive ingredients with proven appetite-reducing effects and the development of functional foods containing them could be useful in managing body weight and preventing overweight/obesity and their related diseases [14]. Several studies have shown that the food industry by-products can be sources of compounds with high nutritional value and potentially valuable bioactive compounds [15,16]. Among these bioactive compounds, dietary fibres and their several degrees of solubility ought to be highlighted. This solubility and the formation of gels in the gastrointestinal tract may help the dietary fibre to reach the colon and be fermented by the gut microbiota, and the products of this fermentation can be associated to different effects on host health and well-being. Among these effects, increasing the colon bacteria biomass, the reduction of colonic pH through the production of SCFA, which are important for enterocytes nutrition and the inhibition of pathogenic bacteria, ought to be highlighted [17]. Dietary fibres from fruit by-products processing have shown to be endowed with the potential to promote an increase in the population of beneficial colonic bacteria such as those from the genera *Lactobacillus* and *Bifidobacterium* [18]. Vieira et al. [19] observed *in vitro* in pure cultures that acerola by-product showed a high selectivity for beneficial strains

over undesirable bacteria and could be used as promising candidate for novel sources of prebiotic ingredients.

The substitution of milk for soluble soy extract in fermented products is described as a promising alternative in the development of new products in Brazil [20]. Several benefits are attributed to the fermentation of soluble soy extract. Among them, the reduction of carbohydrates considered responsible for bloating and flatulence, the increase in the levels of free isoflavones, and the production of bioactive peptides known to confer health benefits to the host [21,22]. Additionally, studies have shown that the consumption of fermented soy-based products may reduce cholesterol concentrations, thereby reducing cardiovascular disease risk [23,24], and other metabolic disorders and diseases associated with obesity. Accordingly, the combination of functional properties of acerola by-product and soluble soy extract with probiotic microorganisms may result in a multi-functional fermented soy product with potential for advantageous changes in the obese human intestinal microbiota. Therefore, the aim of this study was to evaluate the impact of fermented soy probiotic beverages supplemented with acerola (*Malpighia emarginata DC*) by-product on the composition and metabolic activity of human lean and obese microbiota, using the *in vitro* intestinal model TIM-2. The experiments were conducted in order to evaluate the possible benefits that the consumption of fermented soy beverages with acerola by-product may have on the composition and/or activity of the microbiota of obese and lean individuals.

2. METHODS

2.1.Probiotic and starter cultures and acerola by-product origin

Probiotic cultures (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium longum* BB-46) and starter culture (*Streptococcus thermophilus* TH-4) were supplied by Chr. Hansen located at Hørsholm, Denmark. The strains were activated with two successive transfers from stock cultures by inoculation, as followed: *L. acidophilus* LA-5 was activated by inoculation in de Man, Rogosa & Sharpe (MRS) broth (Difco, Le Pont de Claix, France), *B. longum* BB-46 in MRS broth (Difco) containing L-cysteine hydrochloride hydrate (0.05%, w/v, Sigma-Aldrich, Germany) and *S. thermophilus* TH-4 in Hogg-Jago glucose broth (HJ) followed by incubation for 24 h at 37 ºC under aerobic conditions, except for BB-46, which was incubated under anaerobic conditions in an anaerobic chamber (Bactron 600, Sheldon Manufacturing Inc., Oregon, USA). After the second incubation, the inocula were washed twice using saline solution (NaCl, 0.85%, w/v, Sigma-Aldrich) and the cells collected by centrifugation at 13,000

xg for 10 minutes (K243R, Centurion Scientific Ltd., UK), and stored at 4 ºC until the production of the fermented soy beverage took place. The acerola (*Malpighia emarginata* DC) by-product was supplied by a fruit processing company located in São Paulo, Brazil and the resulting by-product flour was obtained as a fine powder (<0.42 mm) as described by Vieira et al. [19].

2.2.Experimental design and fermented soy beverage manufacture

A 2^2 factorial randomized design was employed and four pilot-scale production trials of fermented soy beverage (SF) were produced, in triplicates, in order to evaluate the probiotic combination (*Bifidobacterium longum* BB-46 and *Lactobacillus acidophilus* LA-5), as Factor 1 (PRO = X1), and the acerola by-product powder, as Factor 2 (ABP = X2) in two levels (presence or absence) during manufacture of SF according to **Table 5.1.** All beverages were fermented with the starter culture *Streptococcus thermophilus* TH-4. Each formulation of SF was manufactured in batches of 1 L.

The soy extract powder (200 g/L, Mãe Terra, São Paulo, Brazil) was diluted in distilled water with the assistance of a hand blender (Bosch, Slovenia) and heated in an electric stove under constant agitation. After achieving 50 ºC, sucrose (50 g/L, Jumbo Supermarkten B.V., Veghel, The Netherlands) and dextrose (10 g/L, Roquette, Lestrem, France) were added and mixed with a hand blender for approximately 1 min, and heating continued until achieving 80 ºC, when carrageen gum (1 g/L, ETM 3, AgarGel, São Paulo, Brazil) was added and mixed again until complete dissolution of the gum. When the mixture achieved 90 ºC, it was pasteurized (5 min at 90 ºC). For formulations SF3 and SF4, when the mixture achieved 90 ºC, the acerola by-product powder (20 g/L) was added and the mixture was pasteurized (5 min at 90 ºC) (see **Table 5.1**). After pasteurization, all soy bases were cooled in an ice bath to 37 ºC, for the previously prepared inoculum addition, followed by incubation at 37 ºC in a water bath (SW23, Julabo®, Seelbach, Germany) until achieving pH 5.5. Afterwards, the beverage was cooled and kept at 4 ºC for 2 h, when concentrated acerola juice was added and mixed. Next, the SF were packaged in plastic containers and stored at 4 ºC, until the TIM-2 trials in the 6^{th} and 7^{th} days of storage.

Table 5.1. Variables employed in the production of the fermented soy beverages (SF) studied.

Assay	Factors studied	
	Probiotics combination	Acerola by-product
	X1	*X2*
SF1	-	-
SF2	+	-
SF3	-	+
SF4	+	+

+ = presence - =absence

2.3. *In vitro* evaluation of the effect of the fermented soy beverages on the composition and metabolic activity of the gut microbiota

2.3.1. Lean and obese faeces collection and standardization

Faecal samples were obtained from 5 lean (age range 20-33; 2 males, 3 female) and 13 obese (age range 31-67; 6 males, 7 female) healthy volunteers. Volunteers who donated faeces signed an informed consent form. The individuals were non-smokers and had not used antibiotics, prebiotics, probiotics, or laxatives 3 weeks prior to the donation. The standardized lean and obese microbiota used to inoculate the TIM-2 system was collected and prepared according to Aguirre et al. [25]. The resulting samples were snap-frozen in liquid nitrogen and stored at -80 ºC.

2.3.2. Standard Ileal Efflux Media (SIEM) and dialysate (Dial)

The feeding substrate used for microbiota fermentation in TIM-2 simulates (Standard Ileal Efflux Media – SIEM) was composed of the average non-digestible carbohydrates consumed in a normal western diet. The medium was modified from Gibson et al. [26] for experiments in TIM-2, with a composition of carbohydrates solution [12 $g.L^{-1}$ pectin from citrus (Sigma-Aldrich), 12 $g.L^{-1}$ xylan (Sigma-Aldrich), 12 $g.L^{-1}$ arabinogalactan (Sigma-Aldrich), 12 $g.L^{-1}$ amylopectin (Avebe, Veendam, The Netherlands), 100 $g.L^{-1}$ soluble starch (Fisher Scientific, UK)], 25 g TBCO 6.25x [270 g/L Tween 80, 375 $g.L^{-1}$ bacterial peptone, 375 $g.L^{-1}$ casein, 6.25 $g.L^{-1}$ ox-bile, pH set, 5.8, Tritium Microbiology, Eindhoven, The Netherland], 2 g magnesium sulphate hexahydrate solutions [$MgO_4S.6H_2O$, 50 $g.L^{-1}$, pH set 5.8, Sigma-Aldrich], 2 g L-cysteine HCl solution [20 $g.L^{-1}$, Sigma-Aldrich], 4 g salts [25 $g.L^{-1}$ $K_2HPO_4.3H_2O$, 45 $g.L^{-1}$ NaCl, 4.5 $g.L^{-1}$ $CaCl_2.2H_2O$, 0.05 $g.L^{-1}$ $FeSO_4.7H_2O$, 0.1 $g.L^{-1}$ hemin, Tritium Microbiology]. It does not require pre-digestion and the pH is set to 5.8. Dialysate (dial)

was formulated in a total of 5 L as follow: 500 mL of Dial base solution (25 $g.L^{-1}$, $K_2HPO_4.3H_2O$; 45 $g.L^{-1}$, NaCl; 0.05 $g.L^{-1}$, $FeSO_4.7H_2O$; 0.5 $g.L^{-1}$, ox-bile; Sigma-Aldrich; pH set to 5.8), 50 mL magnesium sulphate hexahydrate solution (described above), 50 mL of calcium chloride solution (45 $g.L^{-1}$, $CaCl_2.2H_2O$), 100 mL of L-cysteine HCl solution (described above), 5 mL of vitamin complex solution (1 $mg.L^{-1}$, menadione; 2 $mg.L^{-1}$, D-biotin; 0.5 $mg.L^{-1}$, vitamin B12; 10 $mg.L^{-1}$, pantothenate; 5 $mg.L^{-1}$, nicotinamide; 5 $g.L^{-1}$, para-aminobenzoic acid; 4 $g.L^{-1}$, thiamine; all from Tritium Microbiology) and distillate water to complete 5 L.

2.3.3. Pre-digestion of the fermented soy beverage

After 6 and 7 days of storage, the SF were submitted to a pre-digestion in 3 steps (gastric, enteric I, and enteric II phases) before their inoculation in TIM-2, carried out according to Buriti et al. [27], with slight modification as follows. Gastric enzymes (gastric pepsin and lipase with final concentration 3 $g.L^{-1}$ and 0.9 $mg.L^{-1}$, respectively, both from Sigma-Aldrich) were added to samples of 25 g of each fermented soy beverage, the pH adjusted with HCl 1 M to 2.0-2.2, and incubated in a water bath (Julabo®) at 37 ºC for 2 h with constant agitation of approximate 150 rpm. After gastric phase simulation, the pH of samples was adjusted to 4.5-4.7 using an alkaline solution pH 12 (NaOH [6 $g.L^{-1}$] and NaH_2PO_4 [10.8 $g.L^{-1}$], Sigma-Aldrich). Bile (pork bile, Sigma-Aldrich) and pancreatin (pork pancreatin, Pancrex powder, Zoetis Belgium SA, Belgium) were added to a final concentration of 10 $g.L^{-1}$ and of 1 $g.L^{-1}$, respectively, and incubated again at 37 ºC for 2 h to simulate enteric phase I. Next, the pH was adjusted to 5.5-5.9 using the alkaline solution described above, containing bile and pancreatin maintained at concentrations of 10 $g.L^{-1}$ and 1 $g.L^{-1}$, respectively, and the samples were incubated for another 2 h at 37 ºC to enteric II phase simulation, reaching a total of 6 h of pre-digestion, with final volume of 52.5 mL to be introduced in TIM-2 units. In the end of the pre-digestions, each sampling shot inoculated in each TIM-2 unit had a mean concentration of live cells of *Streptococcus thermophilus* TH-4 of the 10.19 ± 0.94 log CFU equivalent/portion for all fermented soy beverages. For the probiotic beverages (SF2 and SF4) the *Lactobacillus acidophilus* LA-5 and *Bifidobacterium longum* BB-46 mean viable cell were, respectively, 7.25 ± 1.04 log CFU equivalent/portion and 9.30 ± 0.64 log CFU equivalent/portion. The same condition used for the fermented soy beverages pre-digestion was carried out for 25 g of dial (described above) which was introduced in TIM-2 as the control meal.

2.3.4. TIM-2 experimental protocol

The TIM-2 system (**Figure 5.1**) was described in detail by Maathuis et al. [28]. Before each experiment, TIM-2 units were flushed for 3 h with N_2 prior to the introduction of the microbiota inoculum (30 mL of standardized faecal samples, plus 90 mL of dialysate solution yielding a total of 120 mL total system volume) and it was maintained under this conditions at 37 °C for all assay period with the pH kept at or above 5.9 by automatic titration with 2 M NaOH. The dialysate system, consisting of the semi-permeable hollow membrane through the lumen was responsible for removing excessive volume and fermentation metabolites from the TIM-2 units. For all experiments, the dialysis fluid was set at 1.5 mL/min. The microbiota was adapted for 22 h with standard medium (SIEM) at an introduction rate of 2.5 mL/h. After this adaptation period, a 48 h experimental period was started (**Figure 5.2**). SIEM was added in all TIM-2 units throughout this period, at the same rate as the adaptation period. Two shots of 52.5 mL of pre-digested fermented soy beverage or dial, as the control microbiota (**Table 5.2**), were introduced: immediately after the adaptation period (0h) and after 24 h of fermentation, in each TIM-2 unit (**Figure 5.2**). To simulate the passage of material from the proximal to the distal colon and for the collection of samples for the microbiota and metabolic determinations, total lumen samples of 25 mL were removed from the system, at 0 h (after the adaptation period), 24 h and 48 h of the fermentation assay.

2.4. Metabolic products determinations

2.4.1. Short-chain fatty acids, branched-chain fatty acid and lactate

The samples of lumen and spent dialysis from TIM-2 were analysed for of microbial metabolites. For the organic acids (short-chain and branched-chain fatty acids, and lactate) samples from TIM-2 were centrifuged at 15,700 x*g* for 10 minutes (K243R, Eppendorf, Centurion Scientific, UK), and filtered through a 0.45 μm PFTE filter (Sigma-Aldrich), and diluted where necessary with the mobile phase. Ten microliters were loaded on the column by an autosampler 730 (Metrohm). Molecules were eluted according to their pKa. For this purpose, an ion exclusion chromatography (IEC) using a 883 Ion Chromatograph (IC; Metrohm, Switzerland), equipped with a Transgenomic IC Sep ICE-ION-300 column (30 cm length, 7.8 mm diameter and 7 μm particles) and a MetroSep RP2 Guard. The mobile phase consisted of 1.5 mM aqueous sulphuric acid. A column flow of 0.4 ml/min was used. The temperature of the column was 65 °C. The organic acids were detected using suppressed conductivity

detecting. The analyses of organic acids were performed by Brightlab (Venlo, The Netherlands).

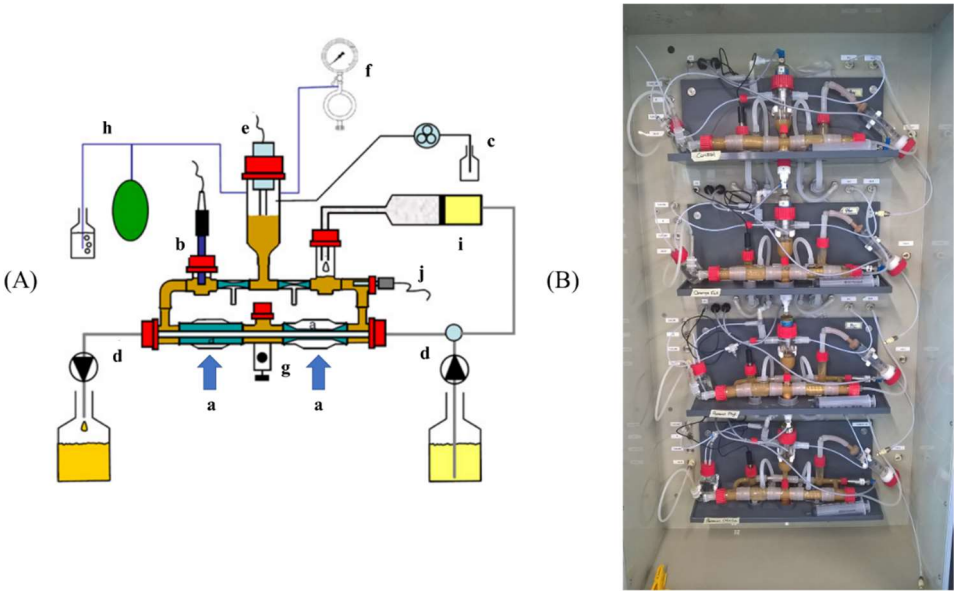


Figure 5.1. (A) Schematic representation of the large intestine (TIM-2 system) from Maathuis et al. [28]. (a) peristaltic compartment containing faecal matter; (b) pH electrode; (c) alkali pump; (d) dialysis liquid circuit with hollow fibre membrane; (e) level sensor; (f) N_2 gas inlet; (g) sampling and shot port; (h) gas outlet; (i) "ileal efflux" container (with SIEM); (j) temperature sensor. (B) Photographs of the cabinet with the 4 units of TIM-2.

Table 5.2. Meal treatment description employed in TIM-2 trials with lean and obese microbiota.

Treatment	Adaptation period	Intervention period
Control	SIEM	SIEM + pre-digested dialysate
SF1	SIEM	SIEM + pre-digested fermented soy beverage without probiotic strains and acerola by-product
SF2	SIEM	SIEM + pre-digested fermented soy beverage with probiotic strains and without acerola by-product
SF3	SIEM	SIEM + pre-digested fermented soy beverage without probiotic strains and with acerola by-product
SF4	SIEM	SIEM + pre-digested fermented soy beverage with probiotic strains and acerola by-product

SIEM - Standard Ileal Efflux Media

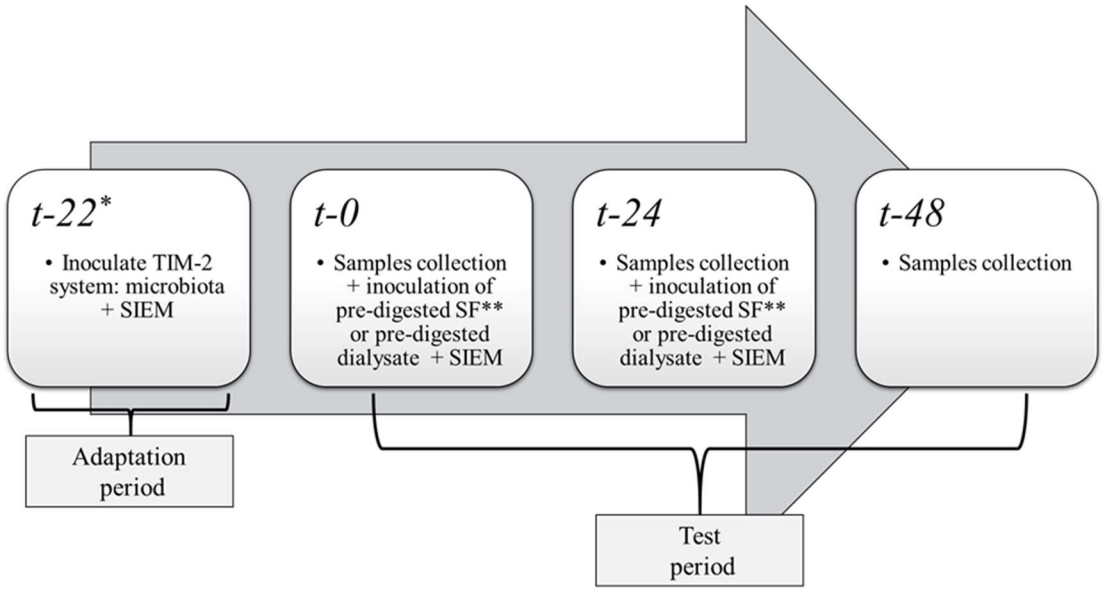


Figure 5.2. Schematic representation of the experimental set up with timeline for the TIM-2 model. **T-22* – Adaptation period of 22 h before the test compound with meal treatments in the TIM-2 model. **See **Table 5.2** for the meal description.

2.4.2. Energy extraction for the host in the form of SFCA and lactate

For the energy extraction for the host in the form of SCFA and lactate, the following values for acetate, propionate, butyrate, and lactate were used for calculation respectively, 874, 1536, 2192 and 1364 kJ mol-1 [29].

2.5. PMA treatment and DNA extractions from TIM-2 samples

The PMA treatment was carried out as described by Nocker et al. [30], with slight modifications as described by Villareal et al. [31]. Aliquots of 200 µL of "lumen" samples collected from TIM-2 at t = 0 h, 24 h and 48 h were washed twice with 500 µL of sterilized Tris EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, TE buffer, pH 8) and centrifuged at 15,700 x*g* for 10 min at 4 ºC (K243R, Eppendorf). The resulting pellet was resuspended in 500 µL of PBS buffer with PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride; Biotium, Inc., Hayward, CA, USA) to a final concentration of 50 mM. Following incubation in an ice bath for 5 min in the dark, duplicate samples were light-exposed for 15 min at a distance of around 20 cm from a 650-W halogen light source (DWE, 650 W, 120 V, GE, Lighting, East Cleveland, OH, USA) in an ice bath, in order to avoid the sample temperature to increase excessively, while mixing for 5 min. After the PMA treatment, the

samples were centrifuged (15,700 *xg* for 10 min at 4 ºC) and the cell pellet was resuspended in 500 µL of TE buffer prior to the DNA isolation procedure, as described below.

For DNA extraction from PMA-treated TIM-2 samples, these samples were transferred to 2 mL centrifuge tubes containing 0.25 g of 0.1 mm zirconia beads (Biospect, Bartlesville, OK, USA) and 3 glass beads (2.5 mm, Biospect), after which 300 µL of Stool Transport and Recovery buffer (STAR buffer, Roche, NJ, USA) were added. The cells were mechanically lysed using a Precellys 24 (Bertin Technologies, USA) at room temperature at 5.5 ms for 3 times of 1 min each. Next, the lysed samples were heated at 95 ºC for 15 min under shaking at 100 rpm in a shaking incubator (VorTempTM 56, Labnet, NJ, USA), after which the samples were centrifuged at 4 ºC for 5 min at 21,000 xg (centrifuge model 5424 R, Eppendorf, Germany). The supernatant was transferred to a new tube of 1.5 mL and the procedure was repeated one more time, but using 200 µL of STAR buffer. For DNA purification, the MaxWell® 16 Tissue Lev Total RNA purification kit (XAS1220, Promega, WI, USA) was employed, as follows: Aliquots of 250 µL of extracted sample were transferred to a MaxWell®16 LEV cartridge rack XAZ1220 and purified in a Maxwell® 16 Instrument (Promega, South Korea) according to Van Lingen et al. [32]. Next, the purified DNA was eluted in 50 µL of Nuclease-Free water (Qiagen, USA). DNA concentration and quality were determined using a DS-11 Microvolume Spectrophotometer (DS11SX, DeNovix, GL Biotech, UK).

2.6. Quantitative Real-Time PCR (qPCR)

The reactions were performed using an ABI-PRISM 7500 sequencing detection system (Applied Biosystems, Bridge-water, NJ, USA). The reaction mixtures (25 µL) contained the PCR Master Mix, each primer at the appropriate concentration (**Table 5.3**), and 5 µL of the template DNA. For determination of *Lactobacillus* spp., the amplification program was: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s, 58 ºC for 20 s, and 72 ºC for 30 s (fluorescence measure) adapted from Rinttilä et al. [33]. On the other hand, the amplification programs used for total bacteria and *Bifidobacterium* spp. were described by Furet et al. [34]. For determination of target species *L. acidophilus* and *S. thermophilus*, amplification programs were described by Tabasco et al. [35] and Falentin et al. [36], respectively. For *B. longum*, the amplification program was adapted from Gueimonde et al. [37] as follow: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s, 65 ºC for 1 min and 30 s. To discriminate the target from the non-target PCR products in SYBR Green qPCR reactions, the melting curve

analysis was carried out after amplification. In order to quantify each target microorganisms and/or group, standard curve were generated by serially 10-fold dilutions of genomic DNA and/or 16S rRNA gene (10^8-10^0 copies per µL, except for total bacteria which was 10^9-10^1 copies per µL) amplified from the respective target strains. For *L. acidophilus,* the 16S rRNA was used, considering strain *L. acidophilus* NCFM as the reference strain, with the number of copies of the16S rRNA gene in *L. acidophilus* genome estimated as four [38]. Finally, the target count was estimated by comparing the samples threshold cycle (*Ct*) and a standard curve *Ct*, with coefficients of efficiency from 89 to 105% and the correlation coefficients (*r2*) between 0.99 and 1.00. Additionally, non-template controls (NTC) samples were carried out in all qPCR runs and tested negative. All assays were performed at least in duplicate for TIM-2 trials and in triplicates for qPCR reactions (a total 6 individual values) and the average values expressed as log CFU equivalent/mL of "lumen" were used for analysis.

2.7. Microbiota analysis and bioinformatics

In In order to microbiota analysis regarding the feacel samples from TIM-2, the Illumina 16S rRNA gene amplicon libraries were generated and sequenced. In short, barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step PCR. 10-25 ng genomic (g)DNA was used as template for the first PCR with a total volume of 50 ul using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers appended with Illumina adaptor sequences. PCR products were purified and the size of the PCR products were checked on Fragment analyzer (Advanced Analytical) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer (Advanced Analytical) and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes. The raw sequencing data produced was processed removing the sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. A quality assessment on the remaining reads was performed using the FASTQC quality control tool version 0.10.0. Quality trimming was applied based on Phred quality scores.

Table 5.3 qPCR reactions information

Target	Standard cultures	Primer and probes	Sequence 5'-3'	Concentration (nM)	Master Mix[3]	Reference
Total bacteria	*Escherichia coli* DH5α™ [1]	F_Bact 1369	CGGTGAATACGTTCCCGG	200	1 x TaqMan	Furet et al. [34]
		R_Prok1492	TACGGCTACCTTGTTACGACTT	200		
		P_TM1389F	6FAM-CTTGTACACACCGCCCGTC-TAMRA	250		
Bifidobacterium spp.	*Bifidobacterium longum* BB-46 [2]	F_Bifid 09c	CGGGTGAGTAATGCGTGACC	200	1 x TaqMan	Furet et al. [34]
		R_Bifid 06	TGATAGGACGCGACCCCA	200		
		P_Bifid	6FAM-CTCCTGGAAACGGGTG-TAMRA	250		
Lactobacillus spp.	*Lactobacillus acidophilus* LA-5 [2]	Lac-F	AGCAGTAGGGAATCTTCCA	500	1x Sybr Green PCR	Rinttilä et al. [33]
		Lac-R	CAC CGC TAC ACA TGG AG	500		
Bifidobacterium longum group	*Bifidobacterium longum* BB-46 [2]	Bif_L_F	TTCCAGTTGATCGCATGGTCTTCT	200	1x Sybr Green PCR	Adapted from Gueimonde et al. [47]
		Bif_L_R	GGCTACCCGTCGAAGCCACG	200		
Lactobacillus acidophilus	16SrDNA from *Lactobacillus acidophilus* LA-5 [2]	Acidfor	AGCGAGCTGAACCAACAGAT	200	1x Sybr Green PCR	Tabasco et al. [35]
		Acidrev	AGGCCGTTACCCTACCAACT	200		
Streptococcus thermophilus	*Streptococcus thermophilus* Th-4[2]	Strep_t_F	GTTCACACTGTGACGGTAGCTT	500	1x Sybr Green PCR	Falentin et al. [36]
		Strep_t_R	GAGCCACAGCCTTTAACTTCAGA	500		

[1]Thermo Fisher Scientific, USA; [2]Chrstian Hansen, Hørsholm, Denmark; [3]Applied Biosystem, USA

To the bioinformatic of data, subsequently sequences were analysed using the QIIME-pipeline version 1.9.1 [39]. Unique sequences were aligned using the "align.seqs" command and an adaptation of the Bacterial SILVA SEED database as a template [40]. In order to ensure that comparable regions of the 16S rDNA gene were analyzed across all reads, sequences that started before the 2.5-percentile or ended after the 97.5-percentile in the alignment were filtered. Potentially chimeric sequences were removed. Sequences were aligned and clustered into operational taxonomic units (OTUs; defined by 97% similarity). A biom table file was generated which was subsequently employed for diversity analysis. Rarefaction curves were computed with the "alpha_rarefaction.py", using Simpson metric and a rarefaction depth value of 1225 sequences. Principal Coordinate Analysis (PCoA) plots were obtained using the "beta_diversity_through_plots.py" command, selecting Unweighted UniFrac as desired metric to generate the distance matrix [39]. The heatmap of the relative abundance were performed in the RStudio® (RStudio, Northern Ave, USA).

2.8. Statistical analysis

The Shapiro-Wilk test was used to verify data normality, and the Bartlett test was used to check assumptions of homoscedasticity. The α-diversity data (PD_whole_tree) of both microbiotas were submitted to the parametric variance analyses (ANOVA) and to means comparison by Tukey test with a significance level of $P<0.05$. The other α-diversity data and also metabolic products, energy extraction and PMA-qPCR data not showing normality and homoscedasticity were submitted to Kruskal-Wallis and to means comparison by the Fisher LSD test with a significance level of $P<0.05$. In order to evaluate the microbiota effect on metabolic compounds, energy extraction, as well as on different microorganisms' target by qPCR assay, data were submitted to the Mann-Whitney U test. Additionally, the short-chain fatty acids, branched-chain fatty acids, and energy extraction data were also submitted to the Mann-Whitney U test in order to evaluate the difference between each fermented soy beverage meal and the control meal treatment for each microbiota independently. The statistical package Statistica 13.0 (StatSoft, Tulsa, OK, USA) was employed, and the results were presented as means ± standard deviation (SD).

3. RESULTS

3.1. Metabolite production

The mean of cumulative short-chain fatty acids (SCFA) and lactate produced during the fermentation TIM-2 trials are shown in **Figure 5.3**. The effects of the microbiota and the

experimental meals (fermented soy beverages), as well as of their combination (microbiota x meals) in the SFCA and lactate cumulative production, were evaluated. Generally, significant differences were observed between the lean and the obese microbiota for acetate ($P<0.001$), propionate ($P<0.05$), and lactate ($P<0.001$) cumulative concentration after 48 h of fermentation in TIM-2 system, with acetate and lactate prevailing for the lean microbiota, whereas propionate predominated for the obese microbiota. In addition, lactate production increased significantly ($P<0.05$) for the meals composed by the probiotics combination (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium longum* BB-46) with acerola by-product (ABP), while the acetate increased significantly ($P<0.05$) in presence of the probiotics without ABP. On the other hand, no significant influence was observed for any of the factors studied regarding the n-butyrate cumulative concentration after 48 h of TIM-2 fermentations, but the meals SF2 and SF3 had significantly higher concentrations in comparison with the control meal (see **Table 5.2** for the meal description) in the lean microbiota.

The ratio of SCFA and lactate production, as well as the energy extraction from SCFA are shown in **Figure 5.4**. Acetate was the SCFA in higher proportion in both, the lean and the obese microbiota. In addition, the lean microbiota presented the highest, even though not significantly higher ($P>0.05$), energy extraction from SCFA (195.79 kJ for the SF4). No significant differences ($P>0.05$) were observed in the energy extraction for the fermented soy beverage meal in both microbiota, but the energy extraction in the TIM-2 units inoculated with fermented soy beverages were significantly higher ($P<0.05$) than that of the control meal in the lean microbiota.

Figure 5.5. shows the cumulative production (mmol) of BCFA (iso-valerate and iso-butyrate) at t48 by the lean and the obese microbiota. The lean and the obese microbiota were significant different ($P<0.001$) for the i-butyrate, i-valerate and total BCFA production after 48 h of fermentation in the TIM 2 system. In the obese microbiota the BCFA production was significantly higher ($P<0.05$) than that in the lean microbiota, but in both microbiota, the meal did not present a significant influence in the BCFA production, since that no difference from the control meal was observed for all BCFA.

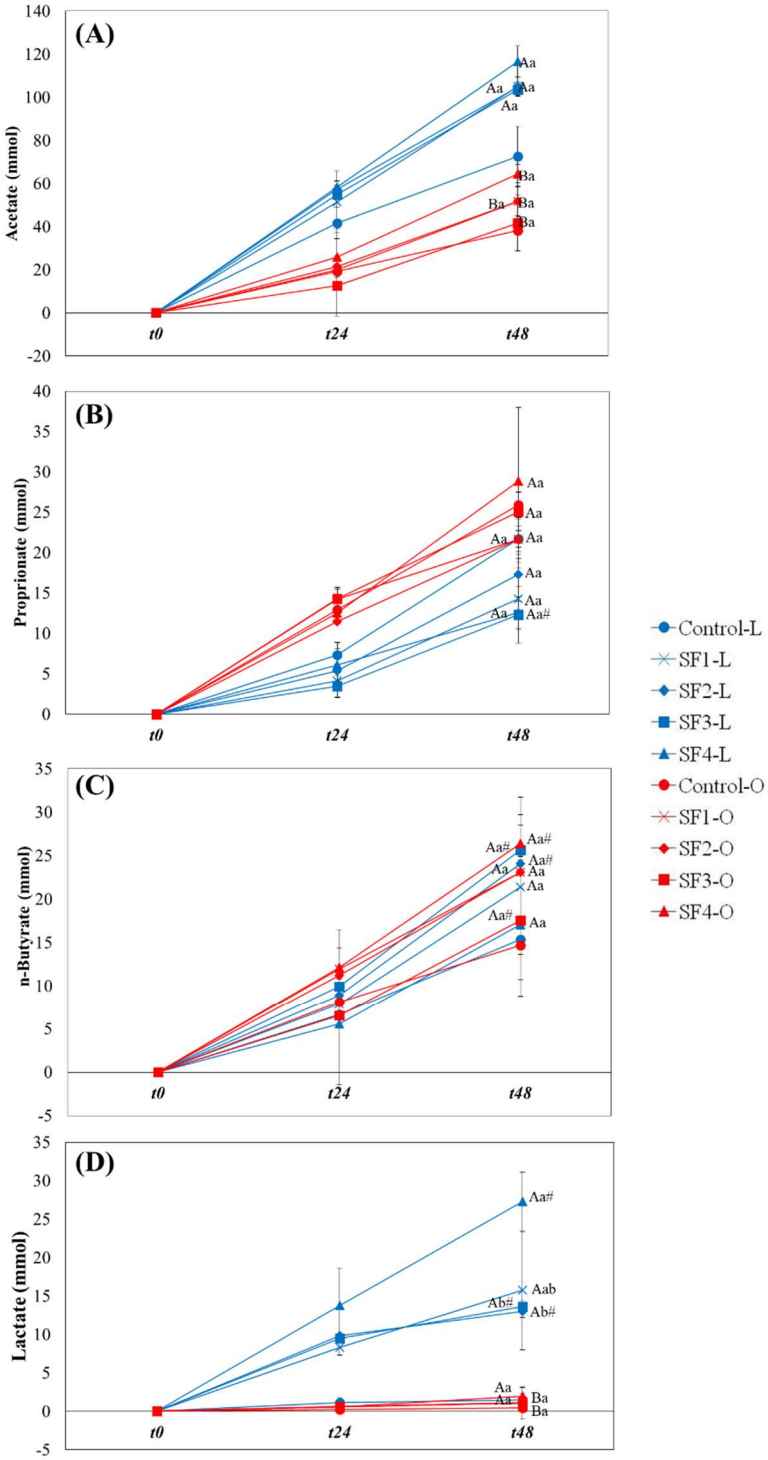


Figure 5.3 Average kinetics of cumulative short-chain fatty acids (SCFA) (A) acetate, (B) propionate and (C) n-butyrate), as well as (D) lactate production (mmol) in the experiment with the lean microbiota (blue) and the obese microbiota (red) for the different meal tested, after the microbiota stabilization (*t0*), 24 h (*t24*), and 48 h (*t48*) of TIM-2 the trial (n=2). The concentration at *t0* was artificially set to zero. [A-B] Different capital letters for each SCFA and/or lactate indicate significant differences ($P<0.05$) in the final cumulative SCFA and/or lactate concentration, between different microbiota in the same meal [a-b] Different lowercase letters for each SCFA and/or lactate indicate significant differences ($P<0.05$) in the final cumulative SCFA and/or lactate concentration, between the different meals for a same microbiota. # Indicate significant differences ($P<0.05$) between the control meal and each treatment meal for the same microbiota. See **Table 5.2** to description of different meals tested.

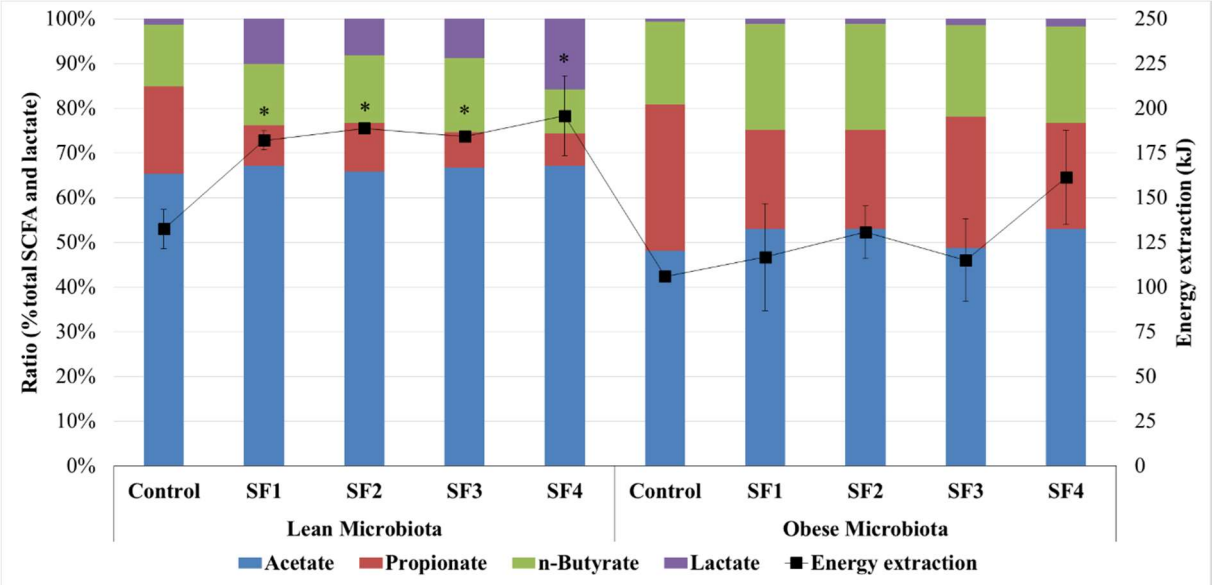


Figure 5.4. Short-chain fatty acids and lactate ratios (%), and energy extraction (kJ) from the different meals at 48 h for the lean and the obese microbiota tested. See **Table 5.2** to description of meals. *Denote significant difference (*P<0.05*) of fermented soy beverages meal and the control meal.

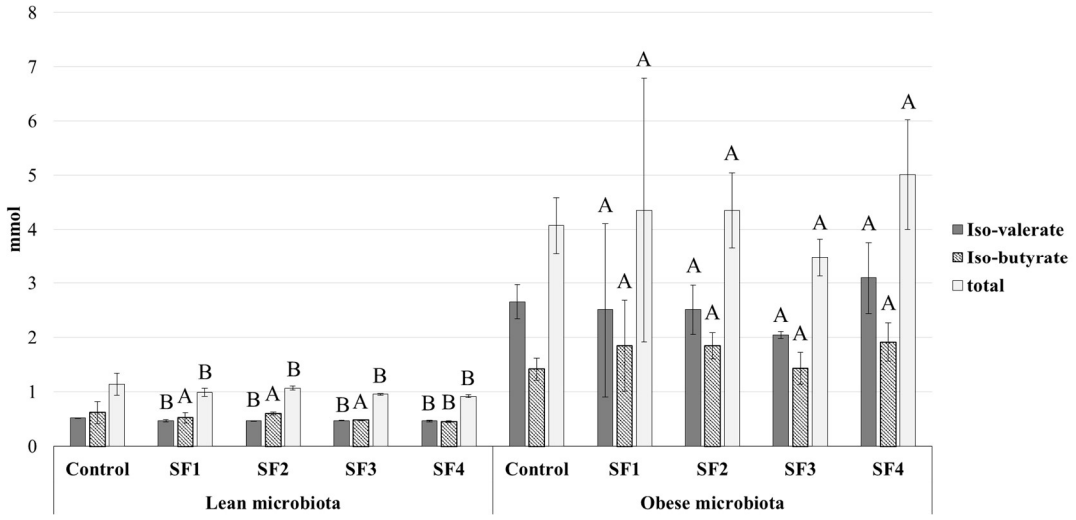


Figure 5.5. Cumulative breach-chain fatty acids (BCFA) production by the lean and the obese microbiotas after 48 h of the experimental meals (n=2). [A-B] Different capital letters for each BCFA indicate significant differences (*P<0.05*) in the final cumulative BCFA concentration, between different microbiota in the same meal.

3.2.Microbial compositional changes

The heatmap with the relative abundance (RA%) for phyla in **Figure 5.6** demonstrates that *Firmicutes, Actinobacteria,* and *Bacteroidetes* were the three most abundance phyla, representing 94.90% up to 99.05% of RA% in the lean microbiota and 96.35 up to 99.15% of RA% in the obese microbiota, generally in the descending order, as follows: *Firmicutes>Actinobacteria>Bacteroidetes*. The changes in the RA% between 0 h and 48 h of

assay of both microbiota employed were observed for these phyla. We observed that in the lean microbiota, *Firmicutes* increased in the presence of meals SF1, SF2, and SF3 with a decrease in *Bacteroidetes* and *Actinobacteria* for SF1. Also, the meal control and SF4 decreased the *Firmicutes* abundance between 0 h and 48 h in the lean microbiota, accompanied by an increase in *Actinobacteria* and, in both meals, a decrease in *Bacteroidetes* in the presence of SF4 and up to 0.85% for the control. In the obese microbiota, *Firmicutes* phyla decreased in all meals, except for the control. Relative abundance of *Actinobacteria* showed increases in all meals in the obese microbiota, accompanied with a decrease or no change in all meals for *Bacteroidetes*, except for the SF4 meal, which presented an increase of 17.95% between 0 h and 48 h of fermentation.

The heatmap of RA% for the bacterial genera present in a proportion of at least 20% of TIM-2 both for the lean and the obese microbiota are shown in **Figure 5.7**. The genera *Bifidobacterium, Collinsella* (*Actinobacteria*), *Bacteroides, Prevotella* (*Bacteroidetes*), *Batua*, and the unclassified genus (g_) of the *Ruminococcaceae* family (*Firmicutes*) were present in both microbiota studied. In general, the *Bifidobacterium* genus and the non-identified genus belonging to the *Ruminococcaceae* family were the most abundant in both microbiota, representing up to 90.95% and 64.95%, respectively, in some samples of the lean and the obese microbiota. In the obese microbiota, the other two genera, the unclassified genus (g_) of the *Lachnospiraceae* family and the genus *Prevotella* were abundant, representing together up to 58.15% in some samples. The genera *Bacteroides* and *Enterococcus* were the next genera more abundant in the lean microbiota after the microbiota stabilization, but after 48 h of assay, a decrease of the RA% of the *Bacteroides* genus in all meals was observed, with a reduction of 26.7% for fermented soy beverage SF3. In addition to *Bacteroides*, the *Enterococcus* RA% decreased between 0h and 48 h of assay, but an increase of 1.0% was only observed in the SF1 meal. Additionally, the genera *Lachnospira, Streptococcus, Eggerthella, [Prevotella], Anaerococcus* and *SMB53*, as well as the non-identified members belonging to the *Bacteridales* order and *SRA202* class were present in lean microbiota and were not observed in the RA% of the obese microbiota, while the genera *Methanobrevibacter, Megasphaera, Lachnospira, Oscillospira, Turicibacter, [Eubacterium], Klebsiella*, and *Bilophila*, as well as non-identified members belonging to the *Lactobacillales* and *Bacteroidales* orders, and *Rikenellaceae, S24-7,* and *Enterobacteriaceae* families were present in the obese RA% and were not shown in the lean microbiota.

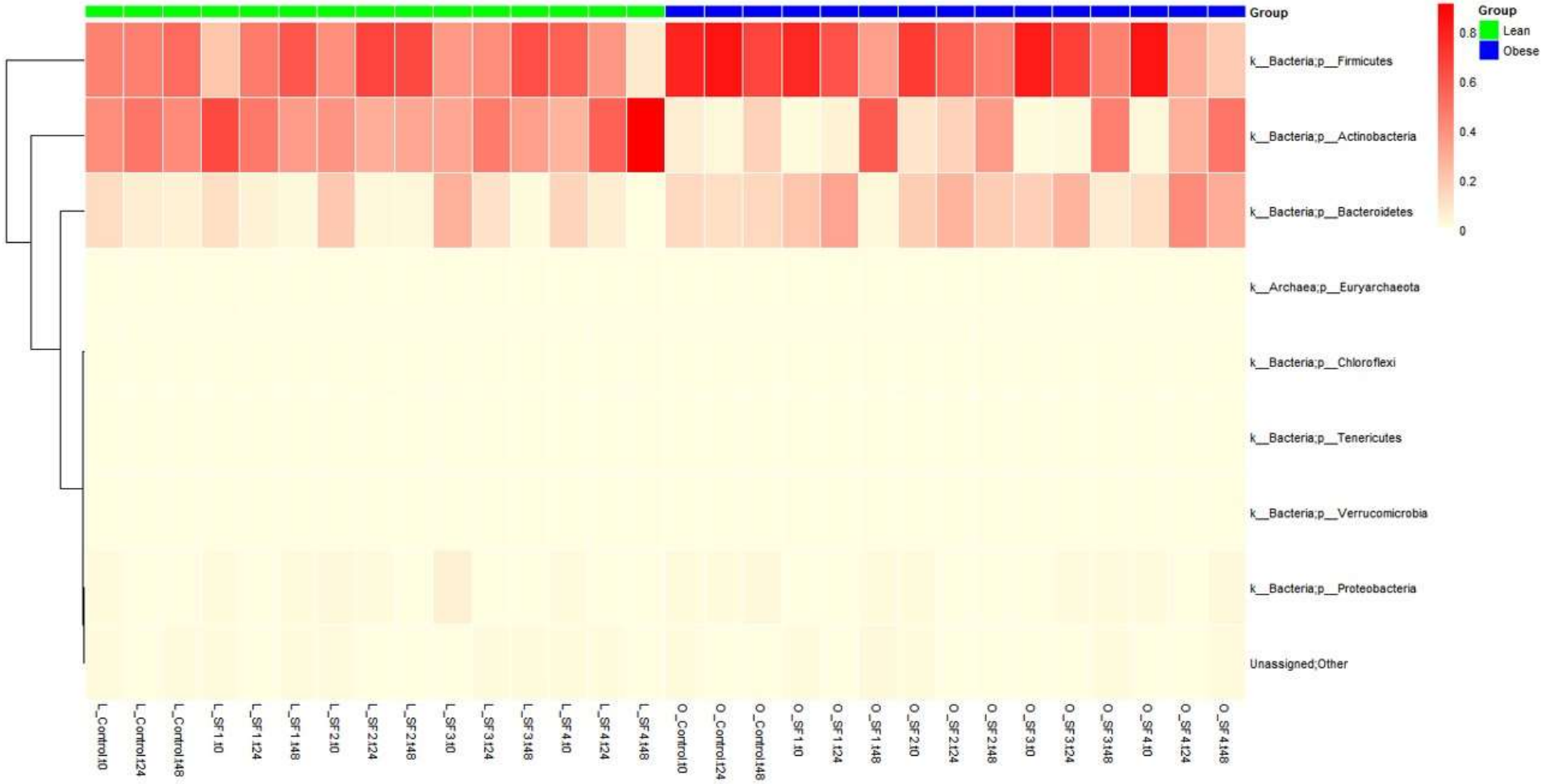


Figure 5.6 Heatmap of relative abundance of the phyla in the lean (green) and obese (blue) microbiota from TIM-2 system, for different meals in sample collection times t=0 h (after the microbiota stabilization), t= 24 h and t=48 h. Control (SIEM + dialysate), SF1 (SIEM + fermented soy beverage without the probiotic strains and the acerola by-product); SF2 (SIEM + fermented soy beverage with the probiotic strains and without the acerola by-product) SF3 (SIEM + fermented soy beverage with the acerola by-product and without the probiotic strains) SF4 (SIEM + fermented beverage soy with the probiotic strains and the acerola by-product). Unassigned phyla were grouped in “Unassigned; Other”

The relative abundance of the *Bifidobacterium* genus in each microbiota was influenced solely by the different fermented soy beverages. The control meal (SIEM + dialysate, see description above) was enough to increase *Bifidobacterium* RA% to 6.7% and to 9.8%, respectively, for the lean and the obese microbiota in the control microbiota. However, the inoculation of fermented soy beverages had a high impact mainly in the obese microbiota, where an increase in the RA% of the *Bifidobacterium* genus to 46.8%, 27.4%, 43.3%, and 45.9% was observed, respectively, for SF1, SF2, SF3, and SF4, while for the lean microbiota, a decrease in the *Bifidobacterium* RA% for SF1 and SF2 (without acerola by-product) was observed, respectively, of 24.0% and of 4.7%, between 0 h and 48 h of fermentation, but the introduction of SF4 increased *Bifidobacterium* RA% to 60.6%, demonstrating that the combination of probiotic strains and acerola by-product could stimulate *Bifidobacterium* mainly in the lean microbiota. A low increase of the *Lactobacillus* genus was observed in some samples of the lean and the obese microbiota, but this corresponded to less than 0.4% of relative abundance. Thus, they were not among the most abundant genera included in the classification as "other".

In general, the lean microbiota diversity was significantly lower ($P<0.001$) than obese microbiota as shown in **Figure 5.8**. The lean microbiota presented a shannon index of 3.722 ± 0.936, while the shannon index for the obese microbiota was 4.622 ± 1.032 (**Figure 5.8A**). Besides, the meal treatments influenced significantly ($P<0.001$) in the diversity of both the lean and the obese microbiotas. Meals with acerola by-product alone (SF3) or in combination of probiotics (SF4) presented a significantly ($P<0.001$) decrease in the microbiota diversity. On the other hand, meal SF2, which presented only probiotic combination, was shown to have the highest general mean of the shannon index (4.776 ± 0.936). In addition, the sampling period of trial (t0, t24 and t48), influenced negatively the diversity in both the lean and the obese microbiotas, which presented significant ($P<0.001$) reductions in the diversity in each subsequent sampling period, with a higher decrease in the diversity in the obese microbiota after t48.

Figure 5.7. Heatmap of relative abundance of the genera (≥20% of presence in the samples) in the lean (green) and obese (blue) microbiota from TIM-2 system, for different meals in sample collection times t=0 h (after the microbiota stabilization), t=24 h, and t=48 h. Control (SIEM + dialysate), SF1 (SIEM + fermented soy beverage without the probiotic strains and the acerola by-product); SF2 (SIEM + fermented soy beverage with the probiotic strains and without the acerola by-product); SF3 (SIEM + fermented soy beverage with the acerola by-product and without the probiotic strains); SF4 (SIEM + fermented beverage soy with the probiotic strains and the acerola by-product). The lower genera presence <20 % of samples were grouped in "Unassigned, Other, Other, Other, Other, Other"

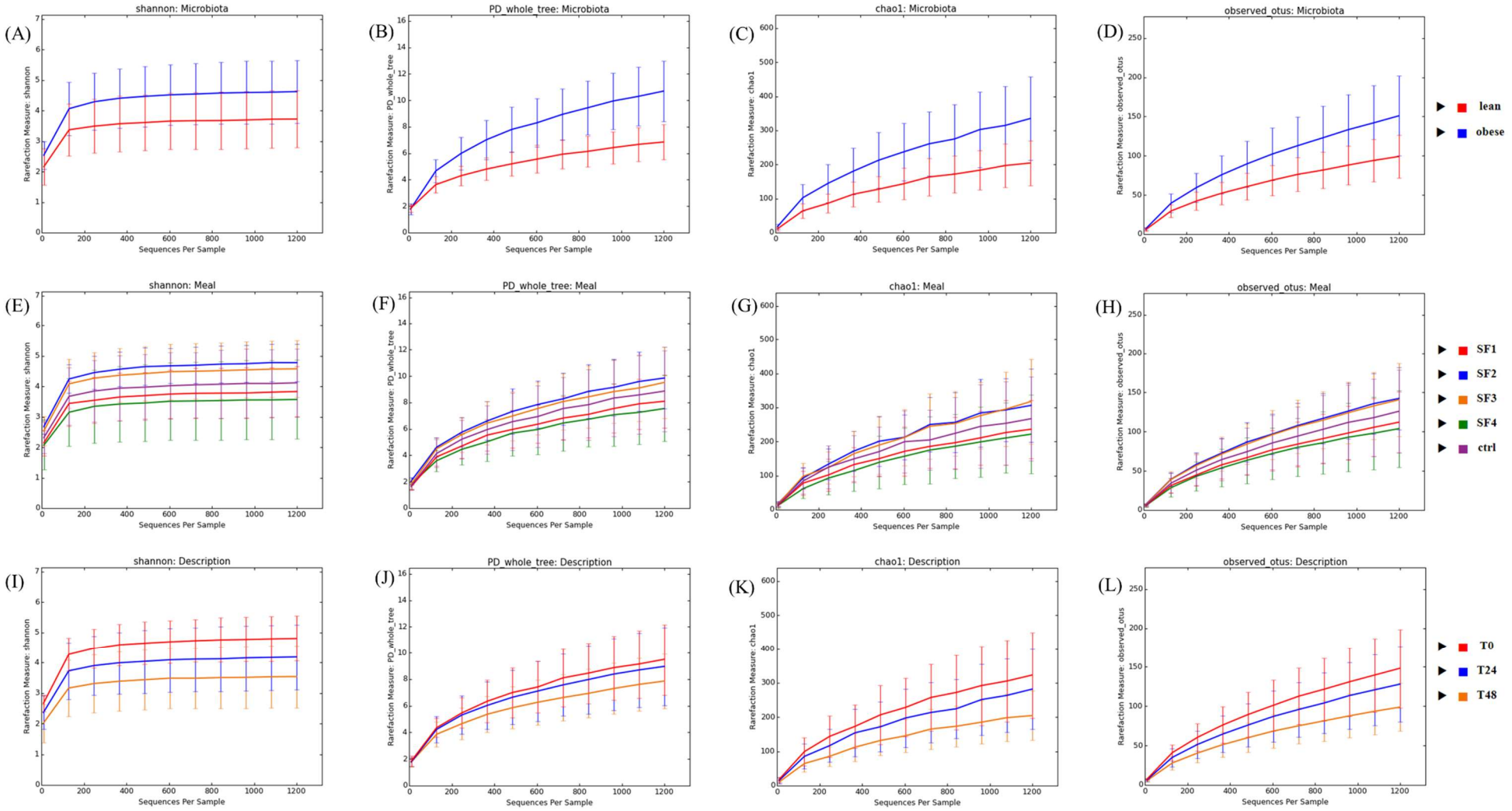


Figure 5.8. Average plots of α-diversity measures (Shanonn index, PD_whole_tree, Chao1, and Observed_OTUs) observed for both the lean and the obese microbiotas (A-D), for the different meal treatments (E-H) and sampling time (I-L) obtained from TIM-2 trials. See **Table 5.2** and **Figure 5.2** for the meal treatment and sampling periods, respectively.

The unweighted PCoA plot of the microbiota composition shows a clear separation between the lean and the obese microbiota (**Figure 5.9A**), which stays separated, even after the various interventions, along the PC1-axis. This indicates that the composition of these two microbiotas was clearly different, as could be observed from the relative abundance of genera. **Figure 5.9C** shows that the microbiota changes over time from T0 (top) to T24 (middle) and to T48 (bottom). These changes could were related with increased *Bifidobacterium* RA% in SF3 and SF4 for lean microbiota and in all meals for the obese microbiota. Even within each microbiota (lean or obese) there are distinct changes observed with each meal (mostly along PC2 and PC3) as observed by combining **Figure 5.9B** and **5.9C**. Compared to the unweighted PCoA, which only looks at the presence or absence of OTUs, the weighted PCoA (**Figures 5.10** and **5.11**) also takes into consideration the amount of the different OTUs. The difference between the lean and the obese microbiotas is less clear than for the unweighted PCoA, but still visible. Comparison of **Figures 5.10A** and **5.10C** shows that after 48 hours of fermentation, obese samples move into the space occupied by the lean microbiota, indicating that it starts to look more alike the lean microbiota, as could be observed by the arrows in **Figure 5.11B**.

3.3. Dynamics of total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., *L. acidophilus, B. longum*, and *Streptococcus thermophilus* in TIM-2 samples assessed by PMA-qPCR

In addition to the Next-Generation Sequencing (NGS) by the Illumina Miseq method, the propidium monoazide (PMA) treatment combined with the real-time quantitative PCR (PMA-qPCR) was employed to evaluate changes in the community of total bacteria and of populations of *Lactobacillus* spp. and *Bifidobacterium* spp. (**Table 5.4**), as well as the target species *L. acidophilus, B. longum,* and *S. thermophilus* (**Table 5.5.**). The results are presented in log CFU equivalent/mL. Generally, the total bacterial community did not show any significant differences ($P>0.05$) between the lean and the obese microbiota studied (**Table 5.4**).

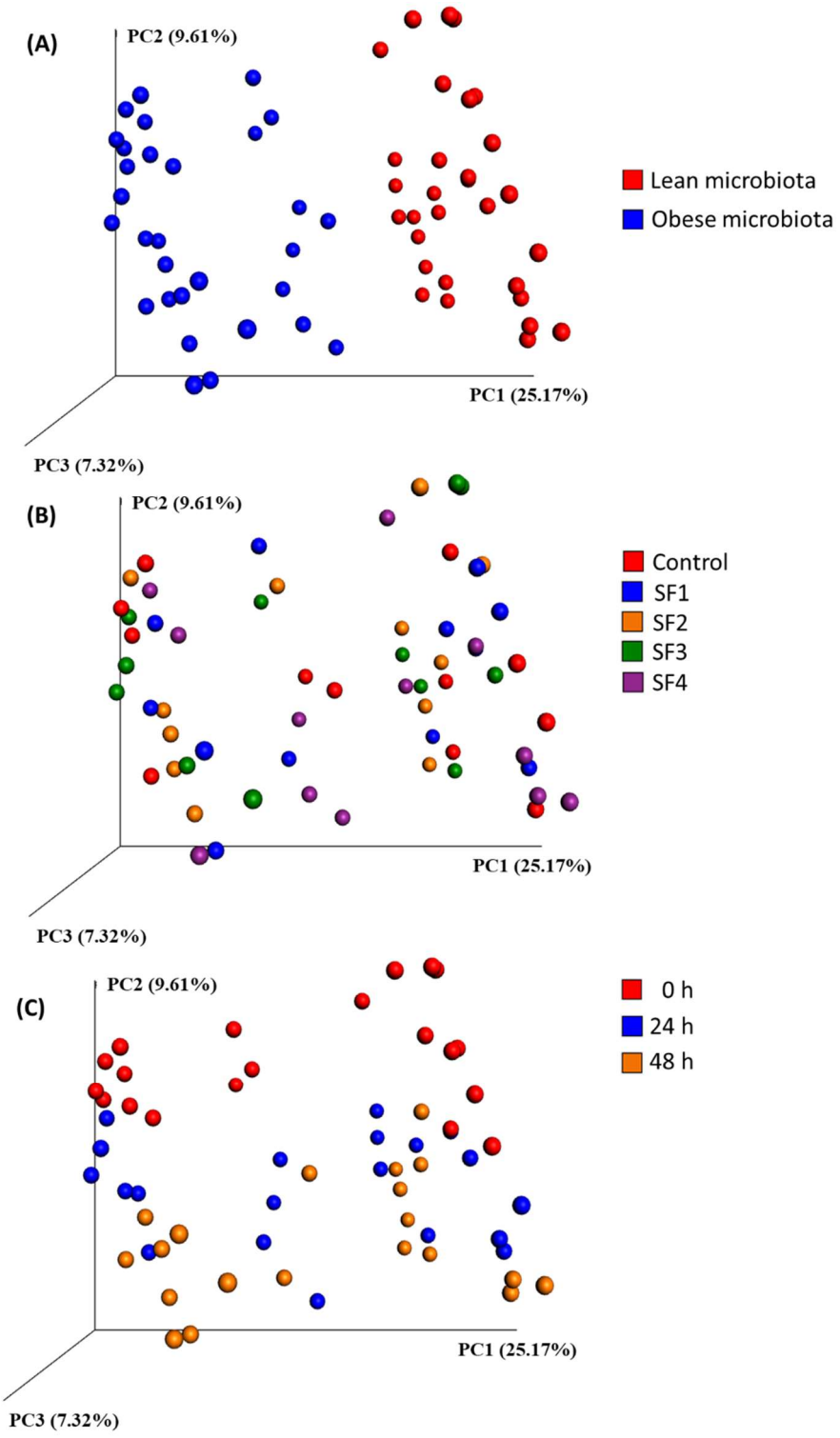


Figure 5.9. Unweighted PCoA. The three principal coordinates from the PCoA analysis of the cosine distances that were calculated between the bacterial compositions of samples. Each colour corresponds to a microbiota group (A), meal (B), and time in hour (C). The variance explained by the PCs is indicated in parentheses on the axes. Control (SIEM + dialysate); SF1 (SIEM + fermented soy beverage without the probiotic strains and the acerola by-product); SF2 (SIEM + fermented soy beverage with the probiotic strains and without the acerola by-product); SF3 (SIEM + fermented soy beverage with the acerola by-product and without the probiotic strains); SF4 (SIEM + fermented beverage soy with the probiotic strains and the acerola by-product).

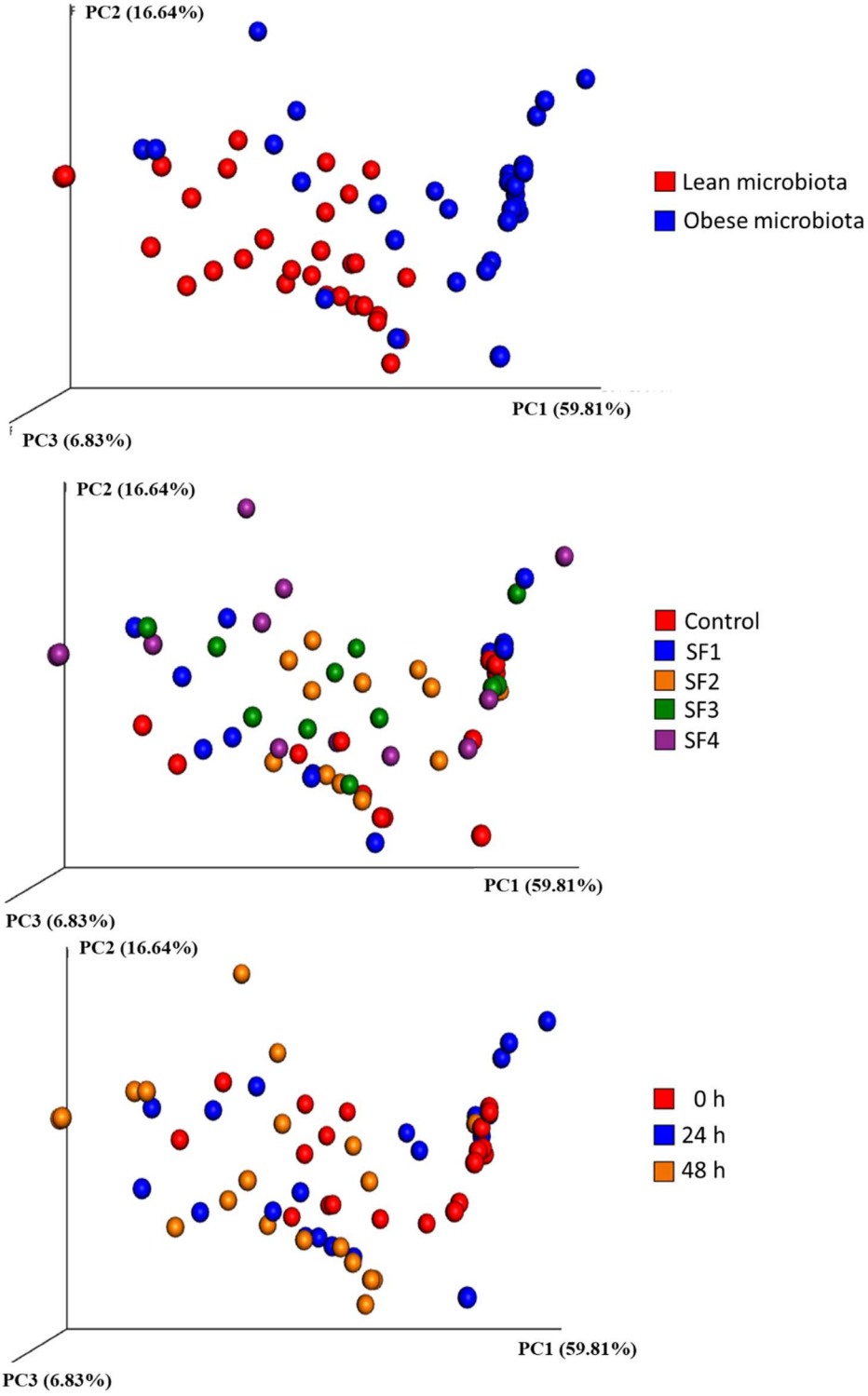


Figure 5.10. Weighted PCoA. The three principal coordinates from the PCoA analysis of the cosine distances that were calculated between the bacterial compositions of samples. Each colour corresponds to a microbiota group (A), meal (B) and time in hour (C). The variance explained by the PCs is indicate in parentheses on the axes. Control (SIEM + dialysate); SF1 (SIEM + fermented soy beverage without the probiotic strains and the acerola by-product); SF2 (SIEM + fermented soy beverage with the probiotic strains and without the acerola by-product); SF3 (SIEM + fermented soy beverage with the acerola by-product and without the probiotic strains); SF4 (SIEM + fermented beverage soy with the probiotic strains and the acerola by-product).

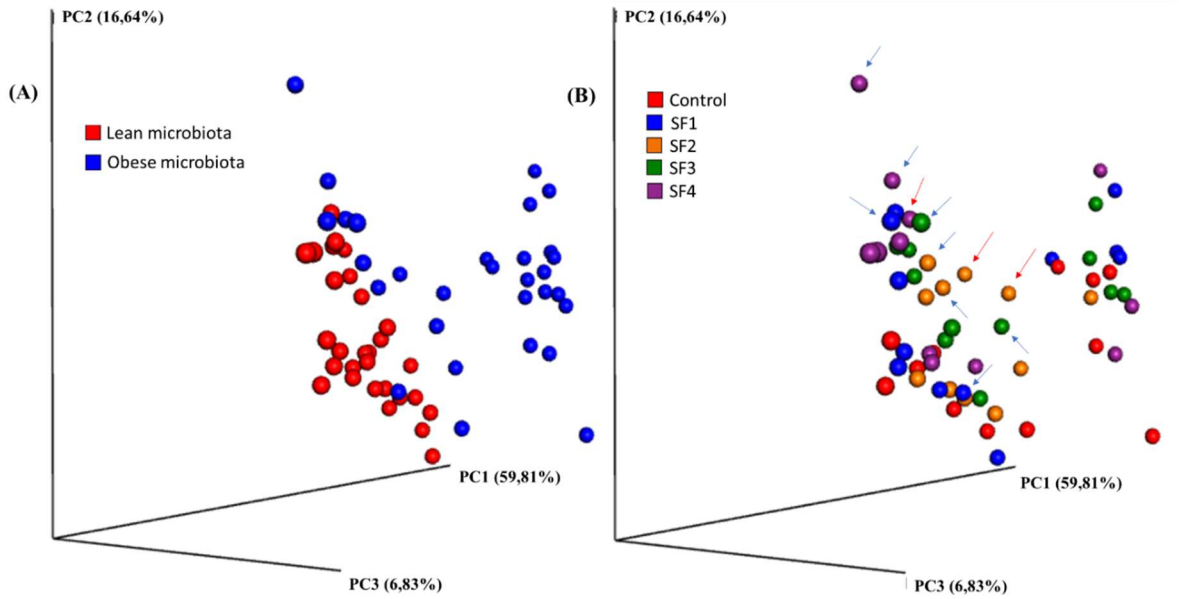


Figure 5.11. Weighted PCoA observed at another angle that observe the sample distribution along the PC3-axis. Each colour corresponds to a microbiota group (A) and meal (B). Red (→) and blue (→) arrows point, respectively, the samples for 24 h and 48 h that move into the space occupied by the lean microbiota. Control (SIEM + dialysate); SF1 (SIEM + fermented soy beverage without the probiotic strains and the acerola by-product); SF2 (SIEM + fermented soy beverage with the probiotic strains and without the acerola by-product); SF3 (SIEM + fermented soy beverage with the acerola by-product and without the probiotic strains); SF4 (SIEM + fermented beverage soy with the probiotic strains and the acerola by-product).

On the other hand, the meal and the time influenced total bacteria community changes significantly ($P<0.05$). No important differences were observed in the total bacteria community for different times and meals, even though some significant differences were obtained ($P<0.05$). In contrast to total bacteria, *Bifidobacterium* spp. and *Lactobacillus* spp. populations were significantly different ($P<0.05$) for microbiota, meal, and time (**Table 5.4**). In general, *Bifidobacterium* spp. populations were higher in the lean microbiota and *Lactobacillus* spp. populations were higher in the obese microbiota. A significant ($P<0.05$) decrease in *Bifidobacterium* spp. populations between 0 h and 48 h of the intervention were observed in all experimental meals, except for meal SF4, in which populations increased during the whole experimental period in the lean microbiota. The highest decrease in *Bifidobacterium* spp. populations was observed for the control meal for the lean microbiota, which presented a reduction of 1.1 log cycle in CFU equivalent/mL between 0 h and 48 h. The combination of the probiotic strains (*B. longum* BB-46 and *L. acidophilus* LA-5) and the acerola by-product in meals SF4 could explain the stability and increase in the *Bifidobacterium* spp. populations. However, this was not observed for meals SF2 and SF3, which presented, respectively, only the probiotics strains and the acerola by-product. We observed that SF2 presented a significant ($P<0.05$) increase in *Bifidobacterium* spp. populations after 24 h, but after 48 h these

populations were lower than those observed for 0 h. The meal SF3 showed a stabe communoty of the *Bifidobacterium* spp. populations during the first 24 h, but after 48 h, as occurred in the case of SF2, these populations decreased significantly ($P<0.05$). Corroborating with what was observed for the lean microbiota, also in the obese microbiota the meal SF4 stimulated a significant ($P<0.05$) increase of approximately 1.5 log cycle of CFU equivalent/mL in *Bifidobacterium* spp. populations between 0 h and 48 h of fermentation. Additionally, the control and the SF1 meals showed significantly ($P<0.05$) increased levels of *Bifidobacterium* spp. populations, but these increases were below 0.3 and 0.8 log cycle of CFU equivalent/mL, respectively, for the control and SF1. The meals SF2 and SF3 were not significantly different ($P>0.05$) at 0 h and at 48 h for *Bifidobacterium* spp. populations for the obese microbiota.

For the lean microbiota, a significant decrease in the *Lactobacillus* spp. populations was observed for all experimental meals, except for SF4. As observed for *Bifidobacterium* spp. populations, the combination of probiotic strains and acerola by-product in SF4 stimulated the *Lactobacillus* spp. populations in the lean microbiota significantly ($P<0.05$). In the first 24 h, the highest *Lactobacillus* populations were observed in the presence of SF4, and after 48 h this increase was approximately 1 log cycle of CFU equivalent/mL, when compared to the population observed at 0 h. Additionally, in the obese microbiota, the meal SF4 increased the *Lactobacillus* spp. populations from the first 24 h, which were thereafter stable until 48 h. The other meals evaluated did not influence the *Lactobacillus* spp. populations between 0 h and 48 h of fermentation significantly ($P>0.05$).

High counts of *Bifidobacterium longum* were observed in both lean and obese microbiotas, with a mean ratio between the *B. longum/Bifidobacterium* spp. of up to 64% in SF1 at 24 h for the lean microbiota and up to 99% in SF3 at 48 h for the obese microbiota (data not shown). Additionally, the *B. longum* populations were significantly lower for the obese microbiota than for the lean microbiota ($P<0.001$). In general, the meal treatments also influenced the *B. longum* populations significantly ($P<0.001$). For the fermented soy beverage SF2, which present only the probiotic combination, lower *B. longum* populations were obtained than control meal, but when in combination with the acerola by-product (in SF4) these populations were significantly higher ($P<0.05$). Besides, in the lean microbiota, the *B. longum* populations decreased significantly ($P<0.05$) between t0 and t48 of the TIM-2 trial, for all the meal treatments, except for SF4 (probiotic combination + acerola by-product, see **Table 5.2**) after 48 h of intervention, which presented a 1 log cycle increase in the *B. longum* populations.

Table 5.4. Total bacteria, *Bifidobacterium* spp., and *Lactobacillus* spp. community obtained by PMA-qPCR in samples from TIM-2.

	Lean Microbiota								
Meal*	**Total Bacteria**			***Bifidobacterium* spp.**			***Lactobacillus* spp.**		
	0 h	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**
Control	10.52 (0.17)^{Baα}	10.42 (0.22)^{Caα}	9.70 (0.16)^{Cbβ}	9.45 (0.20)^{Baα}	9.70 (0.41)^{BCaα}	8.35 (0.57)^{Dbα}	7.80 (055)^{Aaβ}	7.41 (0.85)^{Caβ}	6.49 (0.32)^{Dbβ}
SF1	10.42 (0.01)^{CBaβ}	10.53 (0.09)^{BCaα}	10.23 (0.21)^{Bbβ}	9.72 (0.08)^{ABaα}	9.80 (0.06)^{ABCaα}	9.34 (0.45)^{Bbα}	8.03 (0.16)^{Aabβ}	8.47 (0.10)^{Baβ}	7.78 (0.72)^{Bbβ}
SF2	10.26 (0.17)^{Cbα}	10.73 (0.02)^{ABaα}	10.11 (0.23)^{Bbβ}	9.52 (0.13)^{ABbα}	10.03 (0.07)^{ABaα}	8.93 (0.31)^{Ccα}	8.11 (0.23)^{Aaβ}	8.40 (0.04)^{Baα}	7.22 (0.15)^{Cbβ}
SF3	10.42 (0.10)^{CBaα}	10.57 (0.11)^{BCaα}	10.05 (0.01)^{Bbβ}	9.83 (0.03)^{Aaα}	10.13 (0.14)^{Aaα}	9.30 (0.16)^{Bbα}	8.08 (0.28)^{Aaα}	8.24 (0.30)^{Baα}	7.14 (0.43)^{Cbβ}
SF4	10.80 (0.09)^{Aaα}	10.80 (0.17)^{Aaα}	10.47 (0.17)^{Abα}	9.07 (0.24)^{Ccα}	9.55 (0.75)^{Cbα}	10.00 (0.31)^{Aaα}	8.07 (0.86)^{Acα}	9.55 (0.75)^{Aaα}	8.94 (0.87)^{Abα}
	Obese microbiota								
Meal*	**Total Bacteria**			***Bifidobacterium* spp.**			***Lactobacillus* spp.**		
	0 h	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**
Control	10.50 (0.05)^{Baα}	10.46 (0.15)^{Baα}	10.22 (0.19)^{Cbα}	7.98 (0.09)^{Cbβ}	8.37 (0.17)^{Daβ}	8.31 (0.16)^{Daα}	8.97 (0.23)^{Aaα}	9.06 (0.06)^{Aaα}	9.02 (0.02)^{Aaα}
SF1	11.00 (0.33)^{Aaα}	10.67 (0.04)^{Abα}	10.49 (0.12)^{BCcα}	8.56 (0.32)^{Bbβ}	8.81 (0.05)^{Cbβ}	9.36 (0.11)^{Baα}	8.86 (0.31)^{ABbα}	9.28 (0.14)^{Aaα}	8.87 (0.26)^{Abα}
SF2	10.13 (0.22)^{Cbα}	10.30 (0.24)^{Bbβ}	10.59 (0.17)^{Baα}	9.20 (0.38)^{Aaα}	8.36 (0.50)^{Dbβ}	9.13 (0.41)^{Baα}	8.68 (0.41)^{ABaα}	8.37 (0.08)^{Baα}	8.69 (0.33)^{ABaα}
SF3	10.04 (0.20)^{Ccβ}	10.45 (0.20)^{Bbα}	10.99 (0.18)^{Aaα}	8.54 (0.05)^{Bbβ}	9.71 (0.08)^{Aaβ}	8.71 (0.17)^{Cbβ}	8.48 (0.04)^{Baα}	8.35 (0.29)^{Baα}	8.39 (0.23)^{Baα}
SF4	10.60 (0.11)^{Baβ}	10.37 (0.18)^{Bbβ}	10.33 (0.11)^{Cbα}	8.37 (0.20)^{Bcβ}	9.21 (0.36)^{Bbα}	9.83 (0.20)^{Aaα}	7.86 (0.15)^{Cbα}	9.03 (0.06)^{Aaβ}	8.88 (0.11)^{Aaα}

$^{A-D}$ Different capital letters in a column for each group of microorganisms indicate significant differences ($P<0.05$) between the different meals for a same microbiota and at the same time.
$^{a-c}$ Different lowercase letters in a row for each group of microorganisms indicate significant differences ($P<0.05$) between different times for the microbiota with the same meal.
^{α,β} Distinct Greek letters in a column for each group of microorganisms indicate significant differences ($P<0.05$) between different microbiota for the same meal and the same time.
*****Control = SIEM + dialysate; SF1 = SIEM + fermented soy beverage without the probiotic strains or the acerola by-product; SF2 = SIEM + fermented soy beverage with the probiotic strains but without the acerola by-product; SF3 = SIEM + fermented soy beverage with the acerola by-product but without the probiotic strains; SF4 = SIEM + fermented beverage soy with the probiotic strains and the acerola by-product. Mean of two TIM-2 runs (log CFU equivalents/mL) as calculated from Ct values.

Table 5.5. *Bifidobacterium longum, Lactobacillus acidophilus* and *Streptococcus thermophilus* populations obtained by PMA-qPCR in samples from TIM-2.

Meal*	Lean Microbiota								
	Bifidobacterium longum			***Lactobacillus acidophilus***			***Streptococcus thermophilus.***		
	0 h	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**
Control	9.45 (0.19)$^{Aa\alpha}$	9.73 (0.27)$^{Aa\alpha}$	8.66 (0.40)$^{Bb\alpha}$	2.51 (0.39)$^{A\alpha}$	ND	ND	6.71 (0.26)$^{Ba\alpha}$	4.85 (0.32)$^{Cb\alpha}$	4.80 (0.13)$^{Bb\alpha}$
SF1	9.37 (0.33)$^{Aa\alpha}$	9.57 (0.15)$^{ABa\alpha}$	8.61 (0.74)$^{Bb\alpha}$	2.54 (0.14)$^{Aa\alpha}$	2.65 (0.36)$^{Ba\beta}$	2.66 (0.40)$^{Aa\beta}$	6.90 (0.26)$^{ABa\alpha}$	5.40 (0.70)$^{Bb\alpha}$	5.40 (0.79)$^{Ab\beta}$
SF2	9.00 (0.29)$^{BCb\alpha}$	9.47 (0.04)$^{ABa\alpha}$	8.42 (0.03)$^{Bc\alpha}$	2.94 (0.31)$^{Ab\alpha}$	3.86 (0.55)$^{Aa\alpha}$	2.99 (0.54)$^{Ab\alpha}$	7.28 (0.30)$^{Aa\alpha}$	5.74 (0.28)$^{ABb\alpha}$	5.21 (0.20)$^{Ac\alpha}$
SF3	9.23 (0.06)$^{ABa\alpha}$	9.33 (0.21)$^{Ba\alpha}$	8.76 (0.09)$^{Bb\alpha}$	3.38 (0.24)$^{Aa\alpha}$	2.80 (0.31)$^{Ba\alpha}$	2.55 (0.36)$^{Aa\beta}$	7.08 (0.06)$^{ABa\alpha}$	6.14 (0.31)$^{Ab\alpha}$	5.22 (0.07)$^{Ac\alpha}$
SF4	8.76 (0.16)$^{Cb\alpha}$	9.55 (0.75)$^{ABa\alpha}$	9.79 (0.12)$^{Aa\alpha}$	3.06 (0.71)$^{A\alpha}$	ND	ND	6.02 (0.36)$^{Ca\alpha}$	5.76 (0.22)$^{ABab\alpha}$	5.43 (0.77)$^{Ab\alpha}$
Meal*	**Obese microbiota**								
	Bifidobacterium longum			***Lactobacillus acidophilus***			***Streptococcus thermophilus.***		
	0 h	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**
Control	7.54 (0.07)$^{Ba\beta}$	7.67 (0.28)$^{Ca\beta}$	7.54 (0.40)$^{Ca\beta}$	2.44 (0.35)$^{Ca\alpha}$	2.47 (0.25)^{Ca}	2.45 (0.38)^{Ca}	5.22 (0.29)$^{Ba\beta}$	5.05 (0.45)$^{Ba\alpha}$	4.53 (0.10)$^{Cb\alpha}$
SF1	8.05 (0.36)$^{Ab\beta}$	8.08 (0.11)$^{Bb\beta}$	8.66 (0.09)$^{Aa\alpha}$	2.83 (0.38)$^{BCb\alpha}$	3.97 (0.23)$^{Ba\alpha}$	4.44 (0.36)$^{Ba\alpha}$	6.26 (1.02)$^{Aa\beta}$	5.61 (0.06)$^{Ab\alpha}$	6.15 (0.12)$^{Aa\alpha}$
SF2	8.18 (0.44)$^{Aa\beta}$	7.72 (0.08)$^{Cb\beta}$	8.28 (0.05)$^{Ba\alpha}$	3.41 (0.27)$^{ABa\alpha}$	3.71 (0.37)$^{Ba\alpha}$	2.95 (0.33)$^{Ca\alpha}$	4,74 (0.12)$^{Cb\beta}$	4.81 (0.28)$^{Bb\beta}$	5.46 (0.28)$^{Ba\alpha}$
SF3	7.81 (0.10)$^{Bb\beta}$	8.45 (0.05)$^{Aa\beta}$	8.59 (0.47)$^{ABa\alpha}$	3.39 (0.40)$^{ABb\alpha}$	3.57 (0.33)$^{Bab\alpha}$	4.27 (0.36)$^{Ba\alpha}$	5.51 (0.09)$^{Ba\beta}$	5.10 (0.34)$^{Ba\beta}$	5.28 (0.08)$^{Ba\alpha}$
SF4	7.81 (0.12)$^{Bc\beta}$	8.31 (0.27)$^{ABb\beta}$	8.69 (0.06)$^{Aa\beta}$	3.82 (0.35)$^{Ab\alpha}$	5.76 (0.38)^{Aa}	5.50 (0.28)^{Aa}	6.43 (0.52)$^{Aa\alpha}$	5.53 (0.09)$^{ABb\alpha}$	5.58 (0.51)$^{Bb\alpha}$

$^{A-D}$ Different capital letters in a column for each species indicate significant differences ($P<0.05$) between the different meals for a same microbiota and at the same time.
$^{a-c}$ Different lowercase letters in a row for each species of microorganisms indicate significant differences ($P<0.05$) between different times for the microbiota with the same meal.
$^{\alpha,\beta}$ Distinct Greek letters in a column for each species of microorganisms indicate significant differences ($P<0.05$) between different microbiota for the same meal and the same time.
ND = not determined, values bellow the detection limit (<1.7 log CFU equivalent/mL for *L. acidophilus*)
*Control = SIEM + dialysate; SF1 = SIEM + fermented soy beverage without the probiotic strains or the acerola by-product; SF2 = SIEM + fermented soy beverage with the probiotic strains but without the acerola by-product; SF3 = SIEM + fermented soy beverage with the acerola by-product but without the probiotic strains; SF4 = SIEM + fermented beverage soy with the probiotic strains and the acerola by-product. Mean of two TIM-2 runs (log CFU equivalents/mL) as calculated from Ct values.

In contrast, for the obese microbiota, an increment in the *B. longum* populations were observed in the presence of fermented soy beverage meals SF1, SF3 and SF4. The highest *B. longum* populations were observed in SF4 treatments in both the lean and the obese microbiota, although the meals SF1 and SF3 were not significantly different from SF4 for the obese microbiota. The opposite was observed for the *Lactobacillus acidophilus* populations, which were lower in both lean and obese microbiota, presenting mean *L. acidophilus/Lactobacillus* spp. ratios below 0.2% (data not shown) for both the lean and the obese microbiota in all meal treatments. As observed for the *B. longum* populations, those of *L. acidophilus* were significantly different ($P<0.05$) when the lean and the obese microbiota were compared. In general, *L. acidophilus* populations were significantly higher ($P<0.05$) in the obese microbiota, as was observed for the genus *Lactobacillus*. Additionally, *L. acidophilus* was not detected (<1.7 log CFU equivalent/ml, see **Table 5.5**) after 24 h of intervention for the lean microbiota in the control and SF4 meals, and no significant differences ($P<0.05$) were observed in *L. acidophilus* populations for the other meals between t0 and t48 of trial for the lean microbiota. For the obese microbiota, a significant increase ($P<0.05$) of 1.7 log CFU equivalent/mL in the *L. acidophilus* population was observed between t0 and t48 for SF4. This, was also observed for SF1 (1.6 log CFU equivalent/mL) and SF3 (0.9 log CFU equivalent/mL) for the same microbiota. Generally, significant decreases ($P<0.05$) or no significant changes ($P>0.05$) were observed for the *Streptococcus thermophilus* populations between t0 and t48, in both lean and obese microbiota in all meal treatments. A significant decrease ($P<0.05$) in the *S. thermophilus* population around 2 log CFU equivalent/mL was observed for the lean microbiota, independent of the meal provided. For the obese microbiota, SF2 was the only meal to present a significant increase in the *S. thermophilus* population, whereas SF4 and the control meal presented a significant decrease, as also observed for the lean microbiota. Besides, no significant differences ($P<0.05$) between t0 and t48 were observed for the *S. thermophilus* counts in the TIM-2 interventions with SF1 and SF3, for the obese microbiota.

4. DISCUSSION

4.1. Metabolite production and energy extraction

The fermentation of dietary fibres and protein by the gut microbiota leads to the production of several metabolites. Among these metabolites, the SCFA are the main products to play important roles both on the colonic and the systemic levels. Studies have pointed out that SCFA may promote obesity by increasing the energy absorbed from the colon. However, individually they are neither purely obesogenic nor antiobesogenic, but they are certainly

involved in obesity in some way. SCFA (mainly butyrate) are used by the intestinal cells as energy sources, even when other substrates like glucose and glutamine are present and may affect several physiological processes, while the brain, muscles, and other tissues metabolize acetate, while propionate is metabolized in the liver, interfering in the hepatic cholesterol synthesis [3,41-44]. In addition, recently Fellows et al. [45] demonstrated that these metabolites from the gut microbiota play an important role in the gene expression of epithelial cells. Acetate and lactate production (**Figures 5.3A** and **5.3D**, respectively) by the lean microbiota for all fermented soy beverage meals was much higher than what was produced by the obese microbiota and/or for the control meal in both microbiota. Therefore, the total SCFA and energy extraction (**Figure 5.4**.) were significantly higher ($P<0.05$) for the lean microbiota. These results diverge from those previously reported by Souza et al. [46]. The authors evaluated the effect of cassava bagasse and inulin in lean and obese microbiota, using the same *in vitro* model. The researchers showed that the obese microbiota presented a higher production of acetate and lactate than the lean microbiota for all compounds tested, and consequently a higher energy production by the obese microbiota, although this difference reported was only significant for the inulin treatment. Similarly, Aguirre et al. [47] evaluated the effect of galacto-oligosaccharides (GOS), lactulose, apple fibre, and sugar beet pectin in the same *in vitro* model. They only observed a higher production energy in the form of SCFA by the obese microbiota for lactulose. For GOS the amount of energy was equal for the lean and obese microbiota, while for the two pectins it was lower than fo the lean microbiota. In addition, Aguirre et al. [48] demonstrated that the production of SCFA was significantly higher for the obese microbiota from different volunteers, in comparison with the lean microbiota, after 72 h of daily inoculation with inulin and arabinogalactan in the TIM-2 intestinal *in vitro* model. On the other hand, significantly higher concentrations of acetate were reported by Fukuda et al. [49] in faecal material of mice that received treatment with *Bifidobacterium* strains. Therefore, the higher contents of acetate and lactate in fermented soy beverage meals observed here for the lean microbiota may be related with the high population of *Bifidobacterium* spp. in this microbiota, which were 1 log cycle higher than in the obese microbiota for some meals. High contents of lactate normally do not accumulate in the colon of healthy adult humans, since these compounds may serve as substrates for other bacteria in the production as propionate and butyrate [44], unless fermentation is fast. Some strains belonging to the *Roseburia* genus and certain *Ruminococcaceae*, like *Faecalibacterium prausnitzii*, as well as *Lachnospiraceae* present in many samples of the obese microbiota (See **Figure 5.7**), are able to use acetate and lactate to produce butyrate [50]. Therefore, the lower concentration of lactate and acetate in the obese

microbiota may be related to their consumption by other members of the gut microbiota due to cross-feeding of partial breakdown substances from the complex compounds of the feeding [51].

Souza et al. [46] and Aguirre et al. [48] demonstrated a similar concentration of the branched-chain fatty acids (i-butyrate and i-valerate) as in the present study. Additionally, Souza et al. [46] showed that the obese microbiota produced more BCFA than the lean microbiota, though cassava fermentation by the lean microbiota presented a higher production of BCFA comparing with standard and inulin, while in the present study no significant difference between the fermented soy beverages meals and control was observed. On the other hand, Aguirre et al. [48] shown a higher production of BCFA in both, lean and obese microbiota from arabinogalactan. Besides, the BCFA were significantly correlated with unclassified members of the genera *Faecalibaterium, Bacteroidetes,* and *Enbacterium,* as well as *F. prausnitzii*.

4.2. Microbiota composition changes

In the last decade, many studies reported the beneficial effects of employing probiotic strains, prebiotic fibres, and the combination of both (synbiotics) in several human diseases, including obesity [48,52,53]. The effect of probiotic strains (*Bifidobacterium longum* BB-46 and *Lactobacillus acidophilus* LA-5) and acerola by-products isolated and combined in a food matrix of fermented soy beverage on the composition of lean and obese microbiota, using the sophisticated, dynamic, and validated *in vitro* TIM-2 model, were evaluated in the current study. It has been suggested that the intestinal microbiota is an environmental factor that plays a crucial role in obesity that was traditionally associated with unbalanced energy consumption and expenditure. Obesity, in terms of microbiota, is a complicated unbalance which presents many unclear complications [54]. Diverging from several studies [55-58], in the current experiments the diversity of the obese microbiota was significantly higher ($P<0.05$) than that of the lean microbiota. In a populational study conducted by Le Chatelier et al. [57], the gut microbiota composition and diversity of 123 non-obese and 169 obese Danish individuals were evaluated. The researchers noted that overall adiposity, insulin resistance, and dyslipidaemia were more characterized in individuals presenting a low bacterial richness than in those with a high bacterial richness. Similarly, Cotillard et al. [59] demonstrated that more pronounced dysmetabolism and low-grade inflammation were observed for the individuals with low microbial richness. In addition, the diversity of both microbiotas tested reduced significantly ($P<0.05$) during the TIM-2 interventions. The method of faecal sample standardization used in

the present study is considered as an excellent method for the microbiota preservation and the repeatability of trials in the intestinal *in vitro* model TIM-2, according to Aguirre et al. [25,47]. Also according to the authors, it is similar to fresh faeces, even though the *Bacteroidetes* diversity decreased in all standardization methods we have tested during the intervention in the TIM-2 system.

The human gut microbiota is dominated by the *Bacteroidetes* and *Firmicutes* phyla, but *Actinobacteria, Verrucomicrobia*, and *Proteobacteria* are also found in many people, although in lower proportions [60]. Bengmark [60] reported in a review that *Firmicutes* and *Bacteroidetes* are the two most dominant phyla in the human gut microbiota, with *Firmicutes* and *Bacteroidetes* representing, respectively, 60-80% and 23% of the bacterial composition. However, in the present study with a Dutch-based microbiota, *Firmicutes* and *Actinobacteria* were observed to be the most dominant phyla, representing an overall average of relative abundance of 52.57% and of 31.44%, respectively, for *Firmicutes* and *Actinobacteria*, while the *Bacteroidetes* represented 13.89% of the bacterial composition. Similarly, Zhernakova et al. [61] reported a higher abundance of *Actinobacteria* than of *Bacteroidetes* in a study of the gut microbiome composition and diversity of 1,135 participants from a Dutch-based population. Some studies have suggested that the ratio between *Firmicutes* and *Bacteroidetes* are related with obesity in mice [62,63]. However, Fernandes et al. [64] reported, in a study associating adiposity, gut microbiota, and faecal short chain fatty acids, that the ratio of *Firmicutes* to *Bacteroidetes* was not significantly different between lean and overweight/obese groups. Several possible reasons could be attributed to the contradictory studies, including differences among the subjects studied and the experimental design of the study, with the last one having a high impact on pbserved differences in data. Additionally, the techniques used to study the composition of the microbiota could have contributed to divergent observations. In fact, it has been suggested by the Microbiome Quality Control project described by Sinha et al. [65] that microbial composition estimates could not be compared between studies if sample manipulation and data analysis were not the same [61,66-68].

At the genus level, species within the genera *Bacteroides, Clostridium, Veillonella, Ruminococcus, Eubacterium, Bifidobacterium, Lactobacillus, Fusobacterium, Peptococcus*, and *Peptostreptococcus* were considered as dominating the human gut microbiota [66]. Bianchi et al. [69] reported that fermented vegetable-based beverages supplemented with *Lactobacillus casei* Lc-01 and fructo-oligosaccharides (FOS), in an *in vitro* study of the gut microbiota, increased the beneficial bacteria (*Bifidobacterium* spp. and *Lactobacillus* spp.) populations and reduced detrimental genera, such as *Bacteroides* and *Clostridium* spp. Similar result to those

observed for the present work were reported by the scientific community for studies with prebiotics with proven effects on health and well-being. For example, Canfora et al. [70] evaluated the supplementation of the diet with galacto-oligosaccharides (GOS) in obese pre-diabetic individuals and observed that GOS supplementation increased the abundance of *Bifidobacterium* spp. in faecal samples. Aguirre et al. [48] reported an increase of the genera *Bifidobacterium, Faecalibacterium, Fusicatenibacter,* and *Blautia* after the *in vitro* fermentation of inulin by obese microbiota and a decrease in *Lactobacillus* in the lean microbiota experiments after *in vitro* fermentation of arabinogalactan. Salazar et al. [71] concluded that the consumption of inulin-type fructans selectively modulated *Bifidobacterium* spp. and decreased faecal SCFA concentrations in obese women. Some studies have shown that plant fibres present potential for modulation of the intestinal microbiota, similarly to what was observed in the present study. Gullón et al. [72] evaluated the prebiotic effect of the pseudocereals quinoa and amaranth in the human intestinal ecosystem *in vitro* and reported an increase of the bacterial groups *Bifidobacterium* spp. and *Lactobacillus-Enterococcus* in both vegetable substrates. Souza et al. [46] studied the effect of cassava bagasse on lean and obese microbiota modulation *in vitro* and reported that cassava showed a great increase of *Bifidobacterium* abundance for the lean and the obese microbiota. While in lean microbiota this increase was higher to that observed for the classic prebiotic inulin, for obese microbiota this increase was lower than for inulin.

In contrast with the results described here, Martinez et al. [73] *in vitro* did not observe any significant changes in total bacteria counts throughout the entire intervention with the potential probiotic strain *Lactobacillus amylovorus* DSM 16698 alone or in combination with GOS and *Bifidobacterium animalis* BB-12 using a microbial community of human and of pig origin. Sivieri et al. [74] employed a Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) to evaluate *in vitro* the effect of FOS on the fermentation pattern of the colon microbiota and reported that the administration of FOS to the SHIME® system induced a significant increase in bifidobacteria, lactobacilli, and facultative anaerobes counts in all "colon" compartments. Besides, an increase of *Bifidobacterium* populations in the faecal matter was reported by Ishizuka et al. [75] during *B. animalis* subsp. *lactis* GCL2505 in a randomized clinical study. However, contrasting with the present study regarding the *B. longum* populations, the authors did not observe changes in the *B. lactis* populations. Savard et al. [76] reported an increase in faecal *Lactobacillus* counts of healthy adults which consumed probiotic yoghurt containing *B. animalis* BB-12 and *L. acidophilus* LA-5. As observed for the fermented soy beverage meals in the obese microbiota in the present study, the researchers reported an

increase in the LA-5 populations in the faecal matter after 4 weeks of consumption of the probiotic yoghurt. In addition, Brigidi et al. [77] noted that higher counts of *Streptococcus thermophilus* in probiotic yoghurt than in traditional yoghurt explained the recovery of *S. thermophilus* in the faecal matter. In a later study Mater et al., [78], demonstrated that higher populations of yoghurt cultures (*Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) were recovered from faecal samples of healthy subjects during 12 days of fresh yogurt intake. Saxelin et al. [79] evaluated the effect of yoghurt and cheese food matrixes and capsules on the persistence of probiotic strains in the gastrointestinal tract. The authors concluded that the matrix did not influence the recovery of lactobacilli in faecal samples, but the consumption of cheese resulted in the lowest faecal counts of propionibacteria and bifidobacteria, when compared to the consumption of capsules and yoghurt.

5. CONCLUSION

Overweight, obesity, and diseases caused by these conditions such as type II diabetes and coronary heart diseases may be prevented. Community work and stimulating environmental conditions should stimulate healthier habits, including the consumption of healthier foods and a frequent physical activity practice. These habits would certainly lead to the prevention of overweight and obesity [1]. Nevertheless, these simple changes in people's lifestyle are not always easy to be maintained and patients frequently do not follow their healthcare professional advice. Therefore, it is important to identify successful long term methods for losing weight [48]. Also the gut microbiota has been shown to play a role in overweight/obesity. In the present study, we have identified that different fermented soy beverages, resulted in different effects on lean and obese microbiotas, amongst which a higher production of acetate and lactate for the lean microbiota than for the obese microbiota, which resulted in a higher energy extraction in the lean microbiota, an increase of the relative abundance of the beneficial bacterial genus *Bifidobacterium* in the obese microbiota was observed for all experimental meals, while in the lean microbiota, only the experimental meal supplemented with acerola by-product (SF3) was observed to increase *Bifidobacterium* relative abundance. An increased population of *Lactobacillus* spp. was observed in both microbiotas for fermented soy beverage SF4, which presented the combination of the probiotic strains (*L. acidophilus* LA-5 and *B. longum* BB-46) with the acerola by-product, as well as a maintenance and increase of the *L. acidophilus* population in the obese microbiota. Therefore, a higher diversity observed for the obese microbiota and, after 48 h of intervention, a move of the obese microbiota profile into the lean microbiota profile space was observed through the weighted PCoA, which indicated a

modulation of the obese microbiota by the inoculation of fermented soy beverages, perhaps towards a more healthier composition. The results obtained here confirmed previously conclusions reported by Vieira et al. [19], that acerola by-product could be employed as a promising candidate for novel sources of prebiotic ingredients, since fermented soy beverage supplemented with acerola by-product presented the best characteristics regarding modulation of the obese microbiota, with an increase in beneficial bacteria from the *Bifidobacterium* and *Lactobacillus* genera, as well as after 48 hours of intervention the obese microbiota was apparently more similar to the lean microbiota.

6. REFERENCES

1. World Health Organization-WHO. Obesity and overweight. 2016. http://www.who.int/mediacentre/factsheets/fs311/en/. Accessed 26 Jun 2017.
2. Elli, M, Colombo, O, Tagliabue, A. A common core microbiota between obese individuals and their lean relatives? Evaluation of the predisposition to obesity on the basis of the fecal microflora profile. Med Hypotheses. 2010; 75(4):350–352.
3. Chakraborti, CK. New-found link between microbiota and obesity. World J Gastrointest Phatophysiol. 2015; 6(4):110-119.
4. Tagliabue, A, Elli, M. The role of gut microbiota in human obesity: Recent findings and future perspectives. Nutr Metab Cardiovasc Dis. 2013; 23(3):160–168.
5. Mosca, A, Leclerc, M, Hugot, J.P. Gut microbiota diversity and human diseases: Should we reintroduce key predators in our ecosystem? Front Microbiol. 2016; 7:455.
6. Zhang, X, Zhao, Y, Xu, J, Xue, Z, Zhang, M, Pang, X, Zhang, X, Zhao, L. Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. Sic Rep. 2015; 5:14405.
7. Greiner, T, Bäckhed, F. Effects of the gut microbiota on obesity and glucose homeostasis. Trends Endocrin Metab. 2011; 22(4):117–123.
8. Rajoka, MSR, Shi, J, Mehwish, HM, Zhu, J, Li, Q, Shao, D, Huang, Q, Yang, H. Interaction between diet composition and gut microbiota and its impact on gastrointestinal tract health. Food Sci Human Wellness. 2017; 6:121-130.
9. Scott, KP, Gratz, SW, Sheridan, PO, Flint, HJ, Duncan, SH. The influence of diet on the gut microbiota. Pharmacol Res. 2013; 69:52-60.
10. Jonkers, DMAE. Microbial perturbations and modulation in conditions associated with malnutrition and malabsorption. Best Pract Res Clin Gastroenterol. 2016; 30:161-172.
11. Butel, M-J. Probiotic, gut microbiota and health. Med Mal Infect. 2014; 44(1): 1-8.
12. Bagarolli, RA, Tobar, N, Oliveira, AG, Araújo, TG, Carvalho, BM, Rocha, GZ, Vecina, JF, Calisto, K, Guadagnini, D, Prada, PO. et al. Probiotics modulate gut microbiota and improve insulin sensitivity in DIO mice. J Nutr Biochem. 2017; 50:16-25.

13. Rasmussen, HE, Hamaker, BR. Prebiotics and inflammatory bowel disease. Gastroenterol Clin N Am. 2017; 46:783-795.

14. Moyano, G, Sáyago-Ayerdi, SG, Largo, C, Caz, V, Santamaria, M, Tabernero, M. Potential use of dietary fibre from *Hibiscus sabdariffa* and *Agave tequilana* in obesity management. J Funct Foods. 2016; 21:1–9.

15. Jahurul, MHA, Zaidul, ISM, Ghafoor, K, Al-Juhaimi, FY, Nyam, K-L, Norulaini, NAN, Sahena, F, Mohd Omar, AK. Mango (*Mangifera indica* L.) by-products and their valuable components: a review. Food Chem. 2015; 183:173-180.

16. O'Shea, N, Arendt, EK, Gallagher, E. Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. Innov Food Sci Emerg Technol. 2012; 16:1–10.

17. Gibson, GR. Fibre and effects on probiotics (the prebiotic concept). Clin Nutr Suppl. 2004; 1(2):25–31.

18. Silva, JK, Cazarin CBB, Bogusz Junior, S, Augusto, F, Maróstica Junior, MR. Passion fruit (*Passiflora edulis*) peel increases colonic production of short-chain fatty acids in Wistar rats. LWT - Food Sci Technol. 2014; 59:1252-1257.

19. Vieira, ADS, Bedani, R, Albuquerque, MAC, Biscola, V, Saad, SMI. The impact of fruit and soybean by-products and amaranth on the growth of probiotic and starter microorganisms. Food Res Int. 2017; 97:356-363.

20. Rossi, EA, Cavallini, DCU, Manzoni, MSJ, Rossi, PR. Produtos probióticos e prebióticos à base de soja. In: Saad, SMI, Cruz, AG, Faria, JAF, editors. Probióticos e prebióticos em alimentos: Fundamentos e aplicações tecnológicas. São Paulo: Livraria Varela; 2011. p. 541–563.

21. Liong, MT, Easa, AM, Lim, P-T, Kang, J-Y. Survival, growth characteristics and bioactive potential of *Lactobacillus acidophilus* in a soy-based cream cheese. J Sci Food Agric. 2009; 89(8): 1382–1391.

22. Mondragón-Bernal, O, Rodrigues, MI, Bolini, HMA, Maugeri, F. Optimization of synbiotic fermented food from hydrossoluble soy extract applying experimental design and sensory analysis techniques. J Sens Stud. 2010; 25(3):371–389.

23. Rossi, EA, Vendramini, RC, Carlos, IZ, Oliveira, MG, Valdez, GF. Effect of a new fermented soy milk product on serum lipid levels in normocholesterolemic men. Arch Latinoam Nutr. 2003; 53(1):47–51.

24. Bedani, R, Rossi, EA, Cavallini, DCU, Pinto, RA, Vendramini, RC, Augursto, EM, Abdalla, DSP, Saad, SMI. Influence of daily consumption of synbiotic soy-based product supplemented with okara soybean by-product on risk factors for cardiovascular diseases. Food Res Int. 2015; 73:142-148.

25. Aguirre, M, Eck, A, Koenen, ME, Savelkoul, PHM, Budding, AE, Venema, K. Evaluation of an optimal preparation of human standardized fecal inocula for in vitro fermentation studies. J Microbiol Methods. 2015; 117:78-84.

26. Gibson, GR, Cummings, JH, Macfarlane, GT. Use od three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Appl Environ Microbiol. 1988; 54(11):2750-2755.

27. Buriti, FCA, Castro, IA, Saad, SMI. Viability of *Lactobacillus acidophilus* in symbiotic guava mousses and its survival under *in vitro* simulated gastrointestinal conditions. Int J Food Microbiol. 2010; 137:121-129.

28. Maathuis, A, Hoffman, A, Evans, A, Sanders, L, Venema, K. The effect of the undigested fraction of maize products on the activity and composition of the microbiota determined in a dynamic in vitro model of the human proximal large intestine. J Am Coll Nutr. 2009; 28(6):657–666.

29. Van der Kamp JW, Jones J, Mccleary B, Topping DJ. Dietary Fibre: New Frontiers for Food and Health. Wageningen: Wageningen Academic Publishers; 2010. p. 586.

30. Nocker, A, Cheung, C-Y, Camper, AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods. 2006; 67:310-320.

31. Villarreal, MLM, Padilha, M, Vieira, ADS, Franco, BDGM, Martinez, RCR, Saad, SMI. Advantageous direct quantification of viable closely related probiotic in petit-suisse cheeses under *in vitro* gastrointestinal conditions by propidium monoazide-qPCR. PlosOne. 2013; 8(12):e82102.

32. Van Lingen, HJ, Edwards, JE, Vaidya, JD, Van Gastelen, S, Saccenti, E, Van Den Bogert, B, Bannink, A, Smidt, H, Plugge, CM, et al. Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. Front Microbiol. 2017; 8:a425.

33. Rinttilä, T, Kassinen, A, Malinen, E, Krogius, L, Palva, A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol. 2004; 97:1166-1177.

34. Furet, J-P, Firmesse, O, Gourmelon, M, Bridonneau, C, Tap, J, Mondot, S, Doré, J, Corthier, G. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol Ecol. 2009; 68:351-362.

35. Tabasco, R, Paarup, T, Janer, C, Peláez, C, Requena, T. Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, L. acidophilus, L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermentes milk. Int Dairy J. 2007; 17:1107-1114.

36. Falentin, H, Henaff, N, Bivic, PL, Deutsch, S-M, Parayre, S, Richoux, R, Sohier, D, Thierry, A, Lortal, S, Postollec, F. Reverse transcription quantitative PCR revealed persistency of thermophilic lactis acid bacteria, metabolic activity until the and of the ripening of Emmental cheese. Food Microbiol. 2012; 29:132-140.

37. Gueimonde, M, Debor, L, Tölkkö, S, Jokisalo, E, Salminen, S. Quantitative assessment of faecal bifidobacteria populations by real-time PCR using lanthanide probes. J Appl Microbiol. 2007; 102:1116-1122.

38. Altermann, E, Russell, WM, Azacarate-Peril, MA, Barrangou, R, Buck, BL, McAuliffe, O, Souther, N, Dobson, A, Duong, T, Callanan, M, et al. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. PNAS. 2005; 102(11):3906-3912.

39. Caporaso, JG, Kuczynski, J, Stombaugh, J, Bittinger, K, Bushman, FD, Costello, E, Fierer, N, Peña, AG, Goodrich, JK, Gordon, JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010; 7(5):335-336.

40. Pruesse, E, Quast, C, Knittel, K, Fuchs, BM, Ludwig, W, Peplies, J, Glöckner, FO. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 2007; 35(21):7188-7196.

41. Rahat-Rozenbloom, S, Fernandes, J, Gloor, GB, Wolever, TMS. Evidence of greater production of colonic short chain fatty acids in overweight than lean humans. Int J Obes. 2014; 38(12):1525-1531.

42. Koh, A, De Vadder, F, Kovatcheva-Datchary, P, Bäckhed, F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell. 2016; 165:1332-1345.

43. Romano, A, Koczwara, JB, Gallelli, CA, Vergara, D, Di Bonaventuta, MVM, Gaetani, S, Giudetti, AM. Fats for thoughts: Na update on brain fatty acid metabolism. Int J Biochem Cell Biol. 2017; 84:40-45.

44. Louis, P, Flint, HJ. Formation of propionate and butyrate by human colonic microbiota. Environ Microbiol. 2017; 19(1);29-41.

45. Fellow, R, Denizot, J, Stellato, C, Cuomo, A, Jain, P, Stoyanova, E, Balázsi, S, Hajnády, Z, Liebert, A, Kazakevych, J, et al. Microbiota derived short chain fatty acids promote histone crotonylation in the colon through histone deacetylases. Nat Commun. 2018; 9:105.

46. Souza, CB, Roeselers, G, Troost, F, Jonkers, D, Koenen, ME, Venema, K. Prebiotic effects of cassava bagasse in TNO's in vitro model of the colon in lean versus obese microbiota. J Funct Foods. 2014; 11:210-220.

47. Aguirre, M, Jonkers, DMAE, Troost, FJ, Roeselers, G, Venema, K. In vitro characterization of the impact of different substrates on metabolite production, energy extraction and composition of gut microbiota from lean and obese subjects. PlosOne. 2014; 26:e113864.

48. Aguirre, M, Souza, CB, Venema, K. The gut microbiota from lean and obese subjects contribute differently to the fermentation of arabionogalactan and inulin. PlosOne. 2016; 11(7):e0159236

49. Fukuda, S, Toh, H, Oshima, K, Nakanishi, Y, Yoshimura, K., Tobe, T, Clarke, JM, Topping, DL, Suzuki, T, Taylor, TD, et al. Bifidobacteria can protect form enteropathogenic infection through production of acetate. Nature. 2011; 469:543-549.

50. Kettle, H, Louis, P, Holtrop, G, Duncan, SH, Flint, H. Modelling the emergent dynamics and major metabolites of the human colon microbiota. Environ Microbiol. 2015; 17(5):1615-1630.

51. Belenguer, A, Duncan, SH, Calder, AG, Holtrop, G, Louis, P, Lobley, GE, Flint, HJ. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. Appl Environ Microbiol. 2006; 72(5): 3593-3599.

52. Alard, J, Lehrter, V, Rhimi, M, Mangin, I, Peucelle, V, Abraham, A-L, Mariadassou, M, Maguin, E, Waligora-Dupriet, A-J, et al. Beneficial metabolic effects of selected probiotics on diet-induce obesity and insulin resistance in mice are associated with improvement of dysbiotic gut microbiota. Environ Microbiol. 2016; 18(5):1484-1497.

53. Selma, MV, González-Sarrías, A, Salas-Salvadó, J, Andrés-Lacueva, C, Alasalvar, C, Örem, A, Tomás-Barberán, FA, Espín, JC. The gut microbiota metabolism of pomegranate or walnut ellagitannins yields two urolithin-metabotypes that correlate with cardiometabolic risk biomarkes: Comparison between normoweight, overweight-obsety and metabolic syndrome. Clin Nutr, 2018; 37(3):897-905.

54. Aguirre, M, Venema, K. Does the gut microbiota contribute to obesity? Going beyond the gut feeling. Microorganisms. 2015; 3:213-235.

55. Dao, MC, Clément, K. Gut microbiota and obesity: Concepts relevant to clinical care. Eur J Intern Med. 2018; 48:18-24.

56. Kong, LC, Holmes, BA, Cotillard, A, Habi-Rachedi, F, Brazeilles, R, Gougis, S, Gausserès, N, Cani, PD, Fellahi, S, Bastard, J-P, et al. Dietary patterns differently associate with inflammation and gut microbiota in overweight and obese subjects. PlosOne. 2014; 9(10):e109434.

57. Le Chatelier, E, Nielsen, T, Qin, J, Prifti, E, Hildebrand, F, Falory, G, Almeida, M, Arumugam, M, Batto, J-M, Kennedy, S, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013; 500:541-549.

58. Liao, Y, Peng, Z, Chen, L, Nüssler, A, Liu, L, Yang, W. Deoxynivalenol, gut microbiota and immunotoxicity: A potential approach? Food Chem Toxicol. 2018; 112:342-354.

59. Cotillard, A, Kennedy, S, Kong, LC, Prifti, E, Pons, N, Le Chatelier, E, Almeida, M, Quinquis, B, Levenez, F, Galleron, N, et al. Dietary intervention impact on gut microbial gene richness. Nature. 2013; 500:585-590.

60. Bengmark, S. Gut microbiota, immune development and function. Pharmacol Res. 2013; 69:87-113.

61. Zhernakova, A, Kurilshikov, A, Bonder, MJ, Tigchelaar, EF, Schirmer, M, Vatanen, T, Mujagic, Z, Vila, AV, Falony, G, Vieira-Silva, S, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. Science. 2016; 352(6285):565-569.

62. Ley, RE, Bäckhed, F, Turnbaugh, P, Lozupone, CA, Knight, RD, Gordon, JI. Obesity alters gut microbiota ecology. PNAS. 2005; 102(31):11070-11075.

63. Ley, RE, Turnbaugh, P, Klein, S, Gordon, JI. Human gut microbes associated with obesity. Nature. 2006; 444:a1022.

64. Fernandes, J, Su, W, Rahat-Rozenbloom, S, Wolever, TMS, Comelli, EM. Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. Nutr Diabetes. 2014; 4:e121.

65. Sinha, R, Abnet, CC, White, O, Knight, R, Huttenhower, C. The microbiome quality control project: baseline study design and future directions. Genome Biol. 2015; 26:a275.

66. Hugon, P, Lagler, J-C, Robert, C, Lepolard, C, Papazian, L, Musso, D, Vlalettes, B, Raoult, D. Molecular studies neglect apparently gram-negative populations in the human gut microbiota. J Clin Microbiol. 2013; 51(10):3286-3293.

67. Ottman, N, Smidt, H, De Vos, WM, Belzer, C. The function of microbiota: who is out there and what do they do? Front Cell Infect Microbiol. 2012; 2:a.104.

68. Zhang, C, Zhang, M, Wang, S, Han, R, Cao, Y, Hua, W, Mao, Y, Zhang, X, Wei, C, Zhao, G, et al. Interaction between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J. 2010; 4:232-241.

69. Bianchi, F, Rossi, EA, Sakamoto, IK, Adorno, MAT, de Wiele, TV, Sivieri, K. Beneficial effect of fermented vegetable beverages on human gastrointestinal microbial ecosystem in a simulator. Food Res Int. 2014; 64:43-52.

70. Canfora, EE, van der Beek, CM, Hermes, GDA, Goossens, GH, Jocken, JWE, Holst, JJ, van Eijk, HM, Venema, K, Smidt, H, Zoetendal, EG, et al. Supplementation of diet with galacto-oligosaccharides increases bifidobacteria, but not insulin sensitivity, in obese prediabetic individuals. Gastroenterol. 2017; 153(1):87-97.

71. Salazar, N, Dewulf, EM, Neyrinck, AM, Bindels, LB, Cani, PD, Mahillon, J, De Vos, WM, Thissen, J-P, Gueimonde, M, De Los Reyes-Gavilán, CG, et al. Inulin-type fructans modulate intestinal *Bifidobacterium* species population and decrease fecal short-chain fatty acids in obese women. Clin Nutr. 2015; 34:501-507.

72. Gullón, B, Gullón, P, Tavaria, FK, Yáñez, R. Assessment of the prebiotic effect of quinoa and amaranth in the human intestinal ecosystem. Food Funct. 2016; 14(9):3782-3788.

73. Martinez, RCR, Cardarelli, HR, Borst, W, Albrecht, S, Schols, HA, Gutiérrez, OP, Maathuis, AJH, Franco, BDGM, De Martinis, ECP, Zoetendal, EG, et al. Effect of galactooligosaccharides and *Bifidobacterium animalis* Bb-12 on growth of *Lactobacillus amylovorus* DSM 16698, microbial community structure, and metabolite production in an in vitro colonic model set up with human or pig microbiota. FEMS Microbiol Ecol. 2013; 84(1):110–123.

74. Sivieri, K, Morales, MLV, Saad, SMI, Adorno, MAT, Sakamoto, IK, Rossi, EA. Prebiotic effect of fructooligosaccharide in the simulator of the human intestinal microbial ecosystem (SHIME®Model). J Med Food. 2014; 17(8):894-901.

75. Ishizuka, A, Tomizuka, K, Aoki, R, Nishijima, T, Saito, Y, Inoune, R, Ushida, K, Mawatari, T, Ikeda, T. Effect of administration of *Bifidobacterium animalis* subsp. *lactis* GCL2505 on defecation frequency and bifidobacterial microbiota compositon in humans. J Biosci Bioeng. 2012; 113(5):587-591.

76. Savard, P, Lamarche, B, Paradis, M-E, Thiboutot, H, Laurin, E, Roy, D. Impact of *Bifidobacterium animalis* subsp. *lactis* BB-12 and, *Lactobacillus acidophilus* LA-5-containing yoghurt, on fezal bacterial counts of healthy adults. Int J Food Microbiol. 2011; 149:50-57.

77. Brigidi, P, Swennen, E, Vitali, B, Rossi, M, Matteuzzi, D. PCR detection of *Bifidobacterium* strains and *Streptococcus thermophilus* in feces on human subjects after oral bacteriotherapy and yoghurt consumption. Int J Food Microbiol. 2003; 81:203-209.

78. Mater, DDG, Bretigny, L, Firmesse, O, Flores, M-J, Mogenet, A, Bresson, J-L, Corthier, G. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of heathy vonlunteers consuming yogurt. FEMS Microbiol Lett. 2005; 250:185-187.

79. . Saxelin, M, Lassig, A, Karjalainen, H, Tynkkynen, S, Surakka, A, Vapaatalo, H, Järvenpää, S, Korpela, R, Mutanen, M, Hatakka, K. Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. Int J Food Microbiol. 2010; 144:293-300.

Attachments

1. Attachment: *Ficha do aluno*

05/02/2018

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9133 - 7493379/2 - Antonio Diogo Silva Vieira

Email:	diogovieira@usp.br
Data de Nascimento:	07/01/1986
Cédula de Identidade:	RG - 55.540.913-2 - SP
Local de Nascimento:	Estado do Ceará
Nacionalidade:	Brasileira
Graduação:	Tecnólogo em Alimentos - Instituto Federal de Educação, Ciência e Tecnologia do Ceará - Ceará - Brasil - 2011
Mestrado:	Mestre em Ciências - Área: Tecnologia de Alimentos - Faculdade de Ciências Farmacêuticas - Universidade de São Paulo - São Paulo - Brasil - 2013

Curso:	Doutorado
Programa:	Tecnologia Bioquímico-Farmacêutica
Área:	Tecnologia de Alimentos
Data de Matrícula:	11/10/2013
Início da Contagem de Prazo:	11/10/2013
Data Limite para o Depósito:	08/02/2018
Orientador:	Prof(a). Dr(a). Susana Marta Isay Saad - 11/10/2013 até o presente. Email: susaad@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 11/10/2013
Prorrogação(ões):	120 dias Período de 11/10/2017 até 08/02/2018
Data de Aprovação no Exame de Qualificação:	Aprovado em 24/11/2015
Estágio no Exterior:	Universiteit Maastricht, Países Baixos - Período de 24/06/2016 até 23/02/2017
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 11/10/2013 Prorrogação em 04/08/2017

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 05/02/2018
Impresso em: 05/02/2018 15:49:20

1/2

05/02/2018

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9133 - 7493379/2 - Antonio Diogo Silva Vieira

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBA5752-1/1	Probióticos em Alimentos e Suas Implicações na Saúde Humana	05/11/2013	16/12/2013	60	4	100	A	N	Concluída
Atividade do Programa	Participou da Etapa de Estágio Supervisionado em Docência do Programa de Aperfeiçoamento de Ensino junto à Disciplina FBT0201 Tecnologia de Alimentos do curso de Nutrição, ministrada aos alunos de graduação do curso de Farmácia e Bioquímica da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (1)	01/02/2014	30/06/2014	-	1	100	A	-	-
FBT5700-3/1	Preparo de Artigos Científicos na Área de Tecnologia Bioquímico-Farmacêutica	03/04/2014	04/06/2014	90	6	100	A	N	Concluída
BMH5760-3/3	Biologia das Mucosas Gástrica e Intestinal (Instituto de Ciências Biomédicas - Universidade de São Paulo)	08/04/2014	13/05/2014	60	4	100	A	N	Concluída
FBA5742-2/2	Química e Bioquímica de Alimentos II	31/10/2014	04/12/2014	60	0	-	-	N	Turma cancelada
FBA5706-2/2	Planejamento Experimental para Otimização de Produtos e Processos em Alimentos	26/02/2015	21/06/2015	60	4	93	A	N	Concluída
FBT5738-1/1	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica III	02/03/2015	10/05/2015	30	2	90	A	N	Concluída
FBA5846-2/1	Avaliação de Riscos Microbiológicos em Alimentos	14/09/2015	20/09/2015	30	2	100	A	N	Concluída
FBT5788-1/1	Aplicação de Alimentos Probióticos na Modulação de Imunidade de Mucosas	28/03/2016	17/04/2016	60	4	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	25	27
Estágios:			
Total:	0	25	27

Créditos Atribuídos à Tese: 167

Observações:
1) Créditos atribuídos de acordo com o disposto na Portaria GR-3588 e GR-4391 - PAE, de 31.08.09 e aprovados pela Comissão Coordenadora de Programa, em Sessão de 08/09/2015.

Conceito a partir de 02/01/1997:
A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 05/02/2018
Impresso em: 05/02/2018 15:49:20

2. Attachment: ***Parecer Consubstanciado do CEP. CAAE:50569215.6.0000.0067***

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Impacto da incorporação de cepas probióticas e de subprodutos de frutas em um produto de soja fermentado simbiótico e sobre a composição e a atividade metabólica da microbiota intestinal humana in vitro

Pesquisador: Susana Marta Isay Saad

Área Temática:

Versão: 2

CAAE: 50569215.6.0000.0067

Instituição Proponente: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 1.393.300

Apresentação do Projeto:

O processamento de frutas pelas indústrias de alimentos gera grande quantidade de subprodutos ricos em compostos bioativos que podem ser utilizados para outros fins. No entanto, grande parte dos resíduos produzidos é descartada, gerando problemas ambientais. Uma solução para minimizar o impacto ambiental e agregar valor econômico a esses subprodutos é a sua utilização no desenvolvimento de novos alimentos com potencial funcional. Paralelamente, o consumo de produtos à base de soja é crescente, em virtude dos benefícios à saúde ocasionados pelo seu consumo, aliado à necessidade de novas alternativas para o leite, principalmente devido à intolerância à lactose
e por opção nutricional, como no caso dos vegetarianos. Os benefícios da soja podem, ainda, ser incrementados quando esse alimento é fermentado com micro-organismos probióticos e suplementado com fibras prebióticas, compondo um produto funcional simbiótico. Sendo assim, o presente projeto visa desenvolver um produto de soja
fermentado simbiótico adicionado de subprodutos de frutas e avaliar o impacto desse produto sobre a composição e a atividade metabólica da microbiota intestinal humana, utilizando um modelo que simula as condições intestinais in vitro. Para tanto, o presente projeto será divido em três etapas: (i) Obtenção do pó dos resíduos (casca e sementes) do processamento de suco de

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Plataforma Brasil

laranja, polpa de manga e de acerola e caracterização físico-química e microbiológica dessas farinhas. Ainda nessa etapa, 10 ou mais cepas probióticas serão testadas quanto à sua capacidade de desconjugação sais biliares, atividade proteolítica e fermentabilidade dos subprodutos de frutas obtidos. Estes subprodutos de fruta em pó e as duas cepas probióticas que presentarem as melhores características nos testes anteriores serão utilizados no desenvolvimento do produto fermentado à base de soja (FS); (ii) Desenvolvimento do produto fermentado de soja: para esse fim, será utilizado um delineamento experimental totalmente casualizado do tipo fatorial 23, totalizando 8 ensaios com três repetições cada, tendo como fatores os probióticos (X1 e X2) e o subprodutos de frutas (X3) em dois níveis (presença ou ausência). As diferentes formulações serão avaliadas quanto às suas características microbiológicas, físico-químicas e sensoriais por até 28 dias de armazenamento a 4 °C. Paralelamente, será testada a resistência das culturas probióticas presentes nas diferentes formulações de FS e em cultura pura frente
às condições gastrintestinais simuladas in vitro aos 1, 14 e 28 dias de armazenamento refrigerado. As quatro formulações de FS que apresentarem os melhores resultados nessa etapa serão selecionadas para serem testadas na etapa seguinte do projeto; (iii) Avaliação do impacto das formulações de FS sobre a composição e a atividade
metabólica da microbiota intestinal em um modelo in vitro do intestino grosso (TIM-2), que simula ascondições do lúmen do cólon proximal. Essa etapa será realizada na Maastrincht University (Venlo, Holanda). Serão realizadas coletas de amostras do TIM-2 para a quantificação dos micro-organismos probióticos, Lactobacillus spp.,
Bifidobacterium spp., e bactérias totais, pelo método de PCR quantitativo (qPCR), utilizando-se primers específicos, e para a determinação da concentração de ácidos graxos de cadeia curta, lactato e amônia produzidos durante a passagem do produto pelo modelo de simulação do trato intestinal. Os resultados serão analisados, conforme o delineamento estatístico proposto, utilizando o pacote estatístico Statistica.

Objetivo da Pesquisa:

Objetivo geral

Desenvolver um produto fermentado de soja simbiótico adicionado de subprodutos de frutas e avaliar o impacto desse produto sobre a composição e a atividade metabólica da microbiota intestinal humana, utilizando um modelo intestinal in vitro.

Objetivos específicos

Selecionar os subprodutos industriais do processamento de frutas a serem empregados, com base

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Plataforma Brasil

Continuação do Parecer: 1.393.300

em suas características físico-químicas, microbiológicas, tecnológicas, funcionais e em seu potencial prebiótico;

Selecionar as cepas probióticas a serem empregadas, com base em sua capacidade de desconjugação de sais biliares e em sua atividade proteolíticas;

Elaborar um produto à base de soja fermentado com as cepas probióticas e subprodutos do processamento de frutas previamente selecionados;

Avaliar a viabilidade dos probióticos e da cultura starter nos produtos fermentados de soja, ao longo do armazenamento refrigerado (4±1°C) por até 28 dias;

Avaliar a resistência do probiótico presente na matriz dos fermentados de soja e em cultura pura frente às condições gástricas e entéricas simuladas in vitro e avaliar os efeitos da matriz alimentícia e da adição dos subprodutos de frutas sobre essa resistência;

Avaliar o comportamento do perfil de textura instrumental e dos demais parâmetros físico-químicos e microbiológicos, ao longo do período de armazenamento dos fermentados de soja, assim como a aceitabilidade sensorial;

Avaliar o impacto do produto à base de soja simbiótico com resíduos de frutas na composição e atividade metabólica da microbiota intestinal, utilizando um modelo que simula as condições intestinais in vitro (TIM-2).

Avaliação dos Riscos e Benefícios:

Os riscos desse estudo são mínimos. Pelo fato dos fermentados de soja possuírem, em sua composição, microrganismos que auxiliam na função intestinal, que são ingredientes reconhecidamente seguros e os subprodutos de frutas utilizados nas formulações dos fermentados de soja, tiveram sua segurança microbiológica, além de serem tratados termicamente durante o processamento dos diferentes fermentados de soja, assim os possíveis desconfortos são mínimos. Adicionalmente, não foram encontradas evidências de risco específico ou desconforto relacionado à análise sensorial em estudos deste tipo. Não há nenhum benefício direto. Porém, o voluntário contribuirá para o desenvolvimento de alimentos com características sensoriais adequadas às expectativas de futuros consumidores.

Comentários e Considerações sobre a Pesquisa:

O projeto pretende avaliar a reutilização de subprodutos do processamento de frutas (laranja, manga e acerola) juntamente com derivados de soja e cepas de bactérias probióticas já conhecidas para dar um aumento no valor nutricional e econômico do produto final. O projeto tem um bom embasamento científico, está ligado a linha de pesquisa do pesquisador principal e apresentou um TCLE dentro dos padrões necessários para um ensaio sensorial.

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Considerações sobre os Termos de apresentação obrigatória:

Os termos de apresentação obrigatória foram anexados à plataforma.

Recomendações:

Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Sem pendências ou inadequações.

Considerações Finais a critério do CEP:

Este CEP entende que o projeto pode ser aprovado.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_610376.pdf	28/12/2015 12:59:09		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_diogovieira_rev28122015.pdf	28/12/2015 12:57:17	Susana Marta Isay Saad	Aceito
Outros	Descricao_da_equipe_diogovieira.pdf	27/10/2015 12:15:59	Susana Marta Isay Saad	Aceito
Declaração de Pesquisadores	Declaracao_de_pesquisadorParticipacaoDiogovieira.pdf	27/10/2015 12:14:15	Susana Marta Isay Saad	Aceito
Cronograma	Cronograma_de_execucao.pdf	27/10/2015 12:13:46	Susana Marta Isay Saad	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_de_instituicao_e_infraestrutura.pdf	27/10/2015 12:05:13	Susana Marta Isay Saad	Aceito
Folha de Rosto	Folha_de_rosto.pdf	27/10/2015 11:56:54	Susana Marta Isay Saad	Aceito
Projeto Detalhado / Brochura Investigador	DiogoVieiraProjetoDoutorado_CEP.pdf	16/10/2015 15:26:16	Susana Marta Isay Saad	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Plataforma Brasil

Continuação do Parecer: 1.393.300

SAO PAULO, 22 de Janeiro de 2016

Assinado por:
Mauricio Yonamine
(Coordenador)

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

3. Attachments: ***Laudo das análises microbiológicas das bebidas fermentadas de soja***

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 62/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB1.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 63/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB2.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 64/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB3.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 65/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB4.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 66/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB5.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 67/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB6.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 68/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB7.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental

LAUDO: 69/17
AMOSTRA: Iogurte de Soja
INTERESSADO: Profa. Dra. Susana Saad
Faculdade de Ciências Farmacêuticas

DATA DE RECEBIMENTO: 06/11/17 DATA DE ANÁLISE: 20/11/17
DATA DE EXPEDIÇÃO: 05/12/17
TIPO DE EXAME: Microbiológico
RESPONSÁVEL: M. Landgraf

1. METODOLOGIA

A amostra foi examinada segundo metodologia descrita em American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. DOWNES, F.P.; ITO, K. eds, 4th ed., 2001, 676p.

2. RESULTADOS

DETERMINAÇÕES	VALORES
Coliformes totais (NMP/g)**	<3,0
Coliformes termotolerantes (NMP/g)**	<3,0
Contagem de *Bacillus cereus* (UFC/g)*	<100
Contagem de bolores e leveduras (UFC/g)*	<10
Contagem de *Estafilococos* coagulase positiva (UFC/g)*	<100
Salmonella spp (em 25g)	AUSÊNCIA

* Unidades Formadoras de Colônias por grama
** Número Mais Provável por grama

3. COMENTÁRIOS

A amostra, composta de aproximadamente 200g, estava acondicionada em pote plástico e identificada como: Iogurte de soja FSB8.

4. OBSERVAÇÕES

Os resultados desta análise têm valor restrito e se aplicam tão somente à amostra enviada pelo interessado.

O conteúdo e as conclusões aqui apresentadas são da exclusiva responsabilidade do(s) autor(es) e não refletem, necessariamente, as opiniões da Universidade de São Paulo.

Profa. Dra. Mariza Landgraf

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3656/3657 - Fax (011) 3815.4410 - e-mail: alinutri@edu.usp.br

4. Attachment: *Termo de Consentimento Livre e Esclarecido - TCLE*

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

1. Informações do Participante da Pesquisa

Nome:		
Documento de Identidade (tipo):	Nº.:	Sexo: ( ) M ( ) F
Local de Nascimento:		Data de Nascimento: / /
Endereço:		Nº.:
Complementos:	Bairro:	
Cidade:		Estado:
CEP:	Telefones:	

2. Informações do Responsável Legal (não se aplica)

Nome:		
Documento de Identidade (tipo):	Nº.:	Sexo: ( ) M ( ) F
Local de Nascimento:		Data de Nascimento: / /
Endereço:		Nº.:
Complementos:	Bairro:	
Cidade:		Estado:
CEP:	Telefones:	

3. Título do Projeto de Pesquisa

Impacto da incorporação de cepas probióticas e de subprodutos de frutas em um produto de soja fermentado simbiótico e sobre a composição e a atividade metabólica da microbiota intestinal humana *in vitro*

4. Duração da Pesquisa

2 anos

5. Nome do Pesquisador Responsável

Profª Dra Susana Marta Issay Saad	
Cargo/ Função: Professora Associada	Nº de registro do Conselho Regional: CRF-8 n. 9.541

6. Instituição/Instituições

Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêutica, Universidade de São Paulo

Eu, Dra. Susana Marta Isay Saad (Professora da Faculdade de Ciências Farmacêuticas – FCF-USP), pesquisadora responsável por esse projeto de pesquisa, e Antonio Diogo Silva Vieira (Doutorando da FCF-USP), pesquisador corresponsável que conduz o projeto sob minha orientação, viemos lhe convidar a participar da análise sensorial de uma bebida de soja fermentada probiótica de acerola, suplementada com subproduto industrial de acerola.

O produto a ser avaliado nesta análise sensorial possui 8 (oito) formulações diferentes, constituídas por extrato hidrossolúvel de soja (leite de soja), extrato de soja em pó, açúcar, dextrose, goma carragena, suco concentrado de acerola, subproduto em pó de acerola, fermento lácteo (*Streptococcus thermophilus* TH-4) e microrganismos que contribuem com as funções intestinais (*Lactobacillus acidophilus* LA-5 e *Bifidobacterium longum* BB-46). Todos os ingredientes empregados nas diferentes formulações do fermentado

Página **1** de **3**

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

de soja são de grau alimentício. O produto foi elaborado e acondicionado, de acordo com as Boas Práticas de Fabricação de Alimentos, nos laboratórios do Departamento de Tecnologia Bioquímico-Farmacêutica.

Caso você tenha interesse em participar, acomode-se junto a uma das cabines do Laboratório de Análise Sensorial (Bloco 16).

Para participar desta análise, você: **deve ter entre 18 e 60 anos**, não possuir histórico de manifestação de alergia, intolerância ou outro tipo de restrição (como doença crônica ou tratamento médico com uso de medicamentos que podem interagir com os ingredientes); não deve estar gripado, resfriado ou indisposto ou ter entrado em contato com materiais, alimentos ou cosméticos de cheiro forte. No caso, de você ser do sexo feminino e estiver grávida também não poderá participar, pois, nessas condições, sua capacidade sensorial está alterada e pode comprometer os resultados do experimento. Atendendo a essas condições, você poderá participar da análise sensorial das bebidas de soja fermentadas de acerola suplementadas com subprodutos em pó de acerola.

Você receberá uma amostra e uma ficha de avaliação e intenção de compra. A amostra contém aproximadamente 20 mL. Prove a amostra e registe na ficha sua opinião com relação aos parâmetros sensoriais de sabor, aroma, aparência, textura e aspecto global, assim como sua intenção de compra fazendo um "X" em um lugar na escala hedônica híbrida (0=desgostei extremamente, 5 = nem gostei nem desgostei, 10 = gostei extremamente) e em algum lugar da intenção de compra que variará entre certamente eu não compraria e certamente eu compraria.

Os riscos desse estudo são mínimos. Pelo fato dos fermentados de soja possuírem, em sua composição, microrganismos que auxiliam na função intestinal, que são ingredientes reconhecidamente seguros e os subprodutos de acerola utilizados nas formulações dos fermentados de soja, tiveram sua segurança microbiológica atestada, além de serem pasteurizados durante o processamento dos diferentes fermentados de soja, assim os possíveis desconfortos são mínimos. Adicionalmente, não foram encontradas evidências de risco específico ou desconforto relacionado à análise sensorial em estudos deste tipo.

Não há nenhum benefício direto. Porém, você contribuirá para o desenvolvimento de alimentos com características sensoriais adequadas às expectativas de futuros consumidores.

Caso você não queira continuar participando da pesquisa, a qualquer momento você pode desistir, sem que haja qualquer penalidade. Havendo qualquer dúvida com relação aos procedimentos, riscos e benefícios relacionados à pesquisa você deve comunicar ao grupo de pesquisa a qualquer momento. É assegurado que todas as informações pessoais serão confidenciais, o sigilo e privacidade também são garantidos, mesmo que os resultados sejam publicados em periódicos científicos. Serão adotados cuidados especiais, caso você seja subordinado ou diretamente ligado aos pesquisadores, para que você não se sinta obrigado a participar do estudo

Você não vai ser remunerado, caso não tenha despesas referentes a sua participação na presente pesquisa. Caso haja algum dano relacionado a sua participação, no presente estudo, você poderá ser reembolsado.

Em caso de intercorrências clínicas e reações adversas durante o estudo, você deverá entrar em contato com os responsáveis por esta pesquisa através dos telefones citados abaixo, e será acompanhado a um serviço de saúde do SUS.

Serão assinadas duas vias do Termo de Consentimento Livre e Esclarecido, sendo que, uma delas será recolhida pelo avaliador e a outra pertencerá a você.

Os responsáveis pelo acompanhamento da pesquisa estarão à disposição para contato:
Antonio Diogo Silva Vieira e Susana Marta Isay Saad.
Faculdade de Ciências Farmacêuticas – Departamento de Tecnologia Bioquímico-Farmacêutica. Av. Prof. Lineu Prestes, 580 CEP 05508-000 São Paulo-SP.
Telefone: (11) 98346-1770 ou (11) 3091-2378 ou (11) 3091-2691

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

Consentimento Pós-Esclarecido

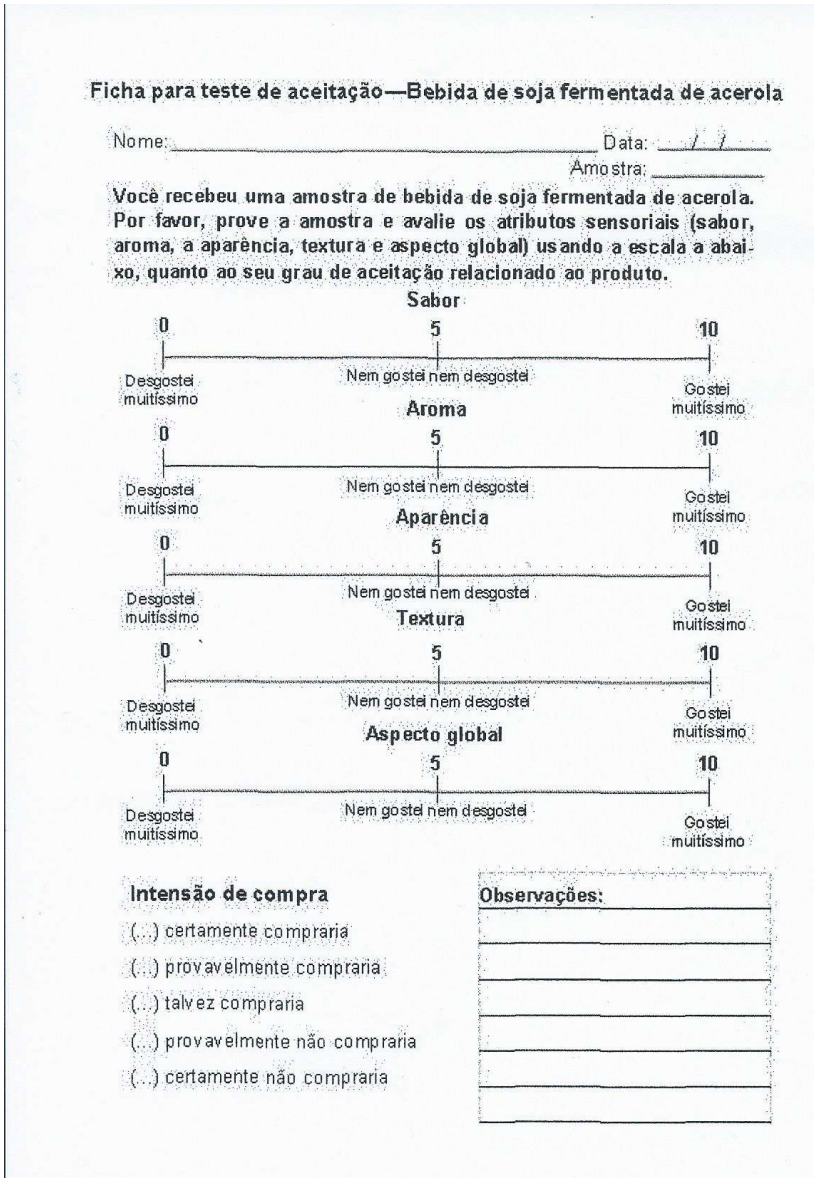
Declaro que, após ter sido convenientemente esclarecido pelo pesquisador e ter entendido o que me foi explicado, consinto em participar do presente Protocolo de Pesquisa.

São Paulo, _____ de _____ de _____.

_____ _____
Assinatura do Participante de Pesquisa Assinatura do Pesquisador Responsável

Para qualquer questão, dúvida, esclarecimento ou reclamação sobre aspectos éticos relativos a este protocolo de pesquisa, favor entrar em contato com o **Comitê de Ética em Pesquisa** da **Faculdade de Ciências Farmacêuticas** da **Universidade de São Paulo**: Av. Prof. Lineu Prestes, 580, Bloco 13 A, Butantã, São Paulo, CEP 05508-000, Telefones 3091-3622 e 3091-3677, e-mail: **cepfcf@usp.br**.

5. Attachment: ***Ficha de avaliação empregada durante os ensaios de análise sensorial.***



Ficha para teste de aceitação—Bebida de soja fermentada de acerola

Nome: _____ Data: ___/___/___

Amostra: _____

Você recebeu uma amostra de bebida de soja fermentada de acerola. Por favor, prove a amostra e avalie os atributos sensoriais (sabor, aroma, a aparência, textura e aspecto global) usando a escala a abaixo, quanto ao seu grau de aceitação relacionado ao produto.

Sabor

0 — 5 — 10

Desgostei muitíssimo | Nem gostei nem desgostei | Gostei muitíssimo

Aroma

0 — 5 — 10

Desgostei muitíssimo | Nem gostei nem desgostei | Gostei muitíssimo

Aparência

0 — 5 — 10

Desgostei muitíssimo | Nem gostei nem desgostei | Gostei muitíssimo

Textura

0 — 5 — 10

Desgostei muitíssimo | Nem gostei nem desgostei | Gostei muitíssimo

Aspecto global

0 — 5 — 10

Desgostei muitíssimo | Nem gostei nem desgostei | Gostei muitíssimo

Intensão de compra

(...) certamente compraria

(...) provavelmente compraria

(...) talvez compraria

(...) provavelmente não compraria

(...) certamente não compraria

Observações: