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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

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Advisor:
Full Professor Susana Marta Isay Saad

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

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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

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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

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. CONCLUSION

The present study showed that, among the vegetable substrates evaluated (orange, acerola, passion fruit, and mango by-products, okara and amaranth flour), orange and passion fruit by-products were the substrates that most promoted the growth of all strains (*Lactobacillus acidophilus* LA-5, *L. fermentum* PCC, *L. reuteri* RC-14, *L. rhamnosus* LGG and GR-1, *L. paracasei* LC431 and F19, *Bifidobacterium animalis* BB-12, *B. longum* BB-46 and BB-02, *Streptococcus thermophilus* TH-4, ST-M6, and TA-40 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, 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 were able to grow well in the presence of raffinose. In addition, *S. thermophilus* TH-4 presented the highest growth in the presence of raffinose among the *S. thermophilus* strains tested. 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 decrease in the sensory acceptance shown for the FSB containing the acerola by-product. Additionally, higher viabilities of probiotic and starter cultures were observed, ranging from 7 to 8 log cfu equivalent/mL and to >9 log cfu equivalent/mL, respectively, during the 28 days of storage and the ABP and the co-cultures did not affect the viability of both probiotic microorganisms (*L. acidophilus* LA-5 and *B. longum* BB-46) and *S. thermophilus*. The incorporation of ABP increased the *B. longum* BB-46 survival significantly to *in vitro* simulated GI conditions, which was not observed for the other microorganisms tested, whereas a significantly ($P<0.05$) decrease of the *B. longum* populations when in co-culture with *L. acidophilus,* and an increase in the *S. thermophilus* populations was observed in presence of *B. longum*. Therefore, the presence of ABP increased the *B. longum* BB-46 survival at 28 days of storage, while this was not observed for the *L. acidophilus* La-5 and *S. thermophilus* TH-4 populations. Moreover, different fermented soy beverages resulted in different effects in the lean and the obese microbiotas. A higher production of acetate and lactate was observed for the lean microbiota than for the obese microbiota, which influenced in the sense of a higher energy extraction in the lean microbiota. Additionally, an increased relative abundance of *Bifidobacterium* spp. in the obese microbiota for all experimental meals, while in the lean microbiota, only the experimental meal supplemented with acerola by-product was observed to increase the *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) and the acerola by-product, as well as a maintenance and an increase in the *L. acidophilus* population in the obese 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.

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