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BIOACESSIBILIDADE, CAPACIDADE ANTIOXIDANTE E POTENCIAL PREBIÓTICO DE SUBPRODUTO DE FRUTAS E HORTALIÇAS

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BIOACESSIBILIDADE, CAPACIDADE ANTIOXIDANTE E POTENCIAL PREBIÓTICO DE SUBPRODUTO DE FRUTAS E HORTALIÇAS

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição na Universidade Federal do Estado do Rio de Janeiro.

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Dedico este trabalho a minha querida avó Maria *in memorian*

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"Se nada ficar destas páginas, algo, pelo menos, esperamos que permaneça: nossa confiança no povo. Nossa fé nos homens e na criação de um mundo em que seja menos difícil amar"

Paulo Freire

RESUMO

O processamento de frutas e hortaliças pela indústria de alimentos gera uma elevada quantidade de resíduos como talos, cascas, sementes, bagaço que são fontes de fibras e compostos bioativos. Neste trabalho, a matéria-prima utilizada foi a farinha obtida do subproduto do processamento integral de frutas e hortaliças (FVBP) com elevado teor de fibras alimentares, carboidratos e proteínas, além de ser fonte de compostos bioativos. No entanto, estes dados não retratam o teor de compostos disponíveis para absorção no intestino delgado, após a digestão gastrointestinal. O teor potencialmente disponível para absorção no intestino pode ser avaliado pela bioacessibilidade, através do modelo de digestão gastrointestinal in vitro. Cabe ressaltar que compostos que não são absorvidos no intestino delgado, atingem o cólon onde podem ser substratos para fermentação pela microbiota intestinal. Neste contexto, o objetivo deste trabalho foi avaliar o teor, perfil e bioacessibilidade de carotenoides, polifenóis e aminoácidos, propriedades antioxidante e prebiótica da farinha FVBP após digestão gastrointestinal in vitro. Os carotenoides, polifenóis (livres e ligados), aminoácidos livres e capacidade antioxidante foram analisados nas etapas inicial, oral, gástrica e intestinal da digestão *in vitro*. A bioacessibilidade, índice de recuperação de polifenóis foram também determinados. O potencial prebiótico da FVBP após o processo de digestão *in vitro* foi determinado a partir de fezes de cinco doadores e avaliado pelo método de PCR quantitativo em tempo real (qPCR) com primers específicos da sequência do gene 16S rRNA. A produção de ácidos graxos de cadeia curta e a citotoxicidade de FVBP foram também determinadas. Os resultados obtidos mostraram aumento do teor de todos os aminoácidos – notadamente dos teores de glutamina e arginina na fase intestinal; flavonoides, carotenoides – especialmente luteína e zeaxantina, e da capacidade antioxidante da FVBP após digestão. Nesta etapa, o índice de recuperação dos polifenóis diminuiu e baixo valor de bioacessibilidade de polifenóis da FVBP (37,7%) foi obtido. A farinha demonstrou potencial prebiótico com impacto positivo no crescimento de *Lactobacillus* e *Bifidobacterium* e promoção da produção de butirato após 24 h de fermentação. Além disso, a FVBP (3%) promoveu o metabolismo de células da linhagem Caco-2 em até 67%. Estes resultados comprovam que a FVBP pode ser usada como alimento ou ingrediente funcional que apresenta elevada capacidade antioxidante e propriedades prebióticas, ou ainda como matéria-prima sustentável para insumos farmacêuticos.

Palavras-chave: resíduos de frutas e hortaliças; compostos funcionais; compostos bioativos; bioacessibilidade; digestão *in vitro*; capacidade antioxidante; propriedade prebiótica.

ABSTRACT

The processing of fruits and vegetables by the food industry generates a high amount of by-products such as stems, peels, seeds, bagasse that are sources of dietary fibers and bioactive compounds. In this work, the flour obtained from the by-product of fruits and vegetables processing (FVBP) with a high content of dietary fibers, carbohydrates, and proteins, in addition to be a source of bioactive compounds was used as the raw material. However, these data do not reflect the content of compounds available for absorption in the small intestine, after gastrointestinal digestion. The content potentially available for absorption in the intestine can be assessed by bioaccessibility, through the *in vitro* gastrointestinal digestion model. It is important to note that compounds that are not absorbed in the small intestine reach the colon where they can be substrate for fermentation by the intestinal microbiota. In this sense, the aim of the present work was to evaluate the content, profile and bioaccessibility of carotenoids, polyphenols and amino acids, antioxidant, and prebiotic properties of FVBP flour after *in vitro* gastrointestinal digestion. Carotenoids, polyphenols (free and bound), free amino acids and antioxidant capacity were evaluated in the initial, oral, gastric and intestinal phases of *in vitro* digestion. Bioaccessibility, polyphenol recovery index, interaction between polyphenols and insoluble fiber fraction were determined. The prebiotic potential of FVBP flour after the *in vitro* digestion was determined with fecal samples of five donors and assessed using the quantitative real-time PCR (qPCR) method with primers specific to the 16S rRNA gene sequence. The production of short-chain fatty acids (SCFAs) and lactate, and the cytotoxicity of FVBP flour were also determined. The results obtained showed an increase of all amino acids content - notably the levels of glutamine and arginine in the intestinal phase; of flavonoids, carotenoids - especially lutein and zeaxanthin, and the antioxidant activity of FVBP flour after digestion. In this phase, the polyphenols recovery index decreased and low polyphenols bioaccessibility value (37.7%) was obtained. The FVBP flour showed potential prebiotic effect with influence on *Lactobacillus* and *Bifidobacterium* growth and promotion of butyrate production after 24 h of fermentation. In addition, FVBP flour (3%) promoted the metabolism of Caco-2 cells by up to 67%. These results demonstrate that FVBP flour can be used as a food or functional ingredient that has high antioxidant capacity and prebiotic properties, or as a sustainable raw material for pharmaceutical ingredients.

Keywords: fruit and vegetable residues; functional compounds; bioactive compounds; bioaccessibility; *in vitro* digestion; antioxidant capacity; prebiotic property.

SUMÁRIO

1. INTRODUÇÃO

A produção de resíduos alimentares ocorre em toda cadeia de alimentos, desde o cultivo até o processamento, varejo e uso doméstico (Mirabella et al., 2014). O resíduo gerado nas etapas desde a pós-colheita até o processamento dos alimentos refere-se à perda de alimentos, enquanto o resíduo gerado nas etapas de varejo, consumo residencial e aquele gerado em serviços de alimentação, refere-se ao desperdício de alimentos (FAO, 2019). Estimativas recentes da FAO indicam que o índice de perda de alimentos, que engloba a perda de alimentos na produção ou cadeia de suprimentos antes de atingir o varejo, é de aproximadamente 14% do total de alimentos produzidos mundialmente (FAO, 2019).

As frutas e vegetais podem representar cerca de 40-50% da perda ou desperdício global de alimentos e, dentre essas perdas ou desperdício, a etapa de processamento representa até 38% do total (Espinosa-Alonso et al., 2020). No Brasil, aproximadamente quase a metade da produção nacional de frutas frescas é voltada para a indústria de processados em geral (SEBRAE, 2015), o que sugere elevada produção de resíduos. Somente na produção de vegetais minimamente processados estima-se que duas unidades de hortifruti, na cidade do Rio de Janeiro, podem gerar anualmente mais de 100.000 kg de resíduos, o que representa cerca de 50% de resíduos oriundos do processamento de frutas e hortaliças (Brito et al., 2020). Os resíduos quando despejados no ambiente, podem apresentar potencial poluente, porém, quando utilizados como matérias-primas ou insumos, são considerados subprodutos e representam uma alternativa viável para reduzir o impacto ambiental destes resíduos. Os subprodutos vegetais englobam todos os resíduos derivados do processamento de frutas e hortaliças como cascas, sementes, caules, folhas, raízes etc. (Espinosa-Alonso et al., 2020). Estes podem ter em sua composição elevada concentração de compostos funcionais como fibra alimentar em subprodutos de manga, goiaba e pêssego (Amaya-Cruz et al., 2015) e em haste, folhas e bagaço de brócolis (Shi et al., 2019), polifenóis em farinha de polpa e casca de caqui (Lucas-González et al., 2018) e carotenoides em bagaço de tomate (Luengo et al., 2014).

Atualmente, visando a redução de produção de resíduos e o desperdício de alimentos, há uma tendência de associar novos modelos comportamentais de consumo e melhorias tecnológicas na redução e valorização dos resíduos alimentares, incluindo a utilização destes como matéria-prima para produção de novos produtos (Morone et al., 2019). Neste sentido, alternativas para utilização destes resíduos, como matéria-prima para formulação de alimentos funcionais (Ferreira et al., 2015) e como potencial nutracêutico Chen et al. (2016) tem sido

descritas. Além disso, a crescente demanda dos consumidores por alimentos saudáveis, com ingredientes naturais, que demonstrem propriedades funcionais, ampliou a busca por matérias-primas alternativas de elevado valor nutricional e de baixo custo, como resíduos vegetais (Bharat Helkar & Sahoo, 2016).

No contexto deste trabalho, e tendo em vista o panorama atual de redução e valorização dos resíduos e da busca por matérias-primas naturais e de baixo custo, os subprodutos gerados no processamento integral de onze espécies de frutas e hortaliças para produção de uma bebida isotônica, foram transformados em uma farinha e seu potencial de uso foi amplamente verificado (Roberta M.S. Andrade et al., 2016; T. B. Brito et al., 2019; Ferreira et al., 2015; Gonçalves et al., 2018). Esta farinha elaborada a partir de subprodutos de frutas e hortaliças (FVBP) tem em sua composição elevado teor de fibra alimentar (48%), principalmente insolúvel (39%), além de quantidade significativa de carboidratos (26%) seguido de proteína (9,5%) (Roberta M.S. Andrade et al., 2016; Ferreira et al., 2015). Além disso, é uma fonte promissora de compostos bioativos, como ácidos fenólicos e flavonoides (Gonçalves et al., 2018) assim como um rico perfil lignocelulósico (Brito et al., 2019), que apresentam efeitos benéficos à saúde já amplamente descritos na literatura (Bacchetti et al., 2019; Slavin, 2013).

No entanto, para exercer um efeito biológico, os compostos funcionais presentes em uma matriz alimentícia complexa precisam ser inicialmente liberados da matriz para, após absorção, estarem disponíveis para exercer sua bioatividade. A digestão pode ter impacto sobre a liberação de compostos de matrizes alimentícias complexas, como a de resíduos vegetais, em função de fatores como mudanças de pH e ação de enzimas digestivas. É importante destacar que a interação entre os compostos na matriz alimentícia, como o aprisionamento físico-químico dos polifenóis pela fibra alimentar, pode também impedir a liberação desses durante a digestão gastrointestinal (A. E Quirós-Sauceda et al., 2014).

Neste sentido, para avaliar o potencial funcional de uma matriz alimentícia complexa, é essencial determinar inicialmente o impacto da digestão gastrointestinal sobre os compostos funcionais presentes, uma vez que o perfil e concentração destes compostos digeridos podem variar em comparação ao da matriz não digerida. Recentemente, o impacto da digestão sobre diversos compostos, como polifenóis, tem sido analisado através da determinação da bioacessibilidade (Lucas-Gonzalez et al., 2016; Rodríguez-Roque et al., 2013; Sun et al., 2019). Esta pode ser definida como a fração de um composto liberado da matriz alimentícia no trato gastrointestinal e disponível para absorção intestinal (Tagliazucchi et al., 2010).

Os estudos de bioacessibilidade *in vivo* são considerados de alto custo e de tempo prolongado. Com isso, estudos *in vitro* têm sido amplamente utilizados por serem mais rápidos, de menor custo e sem restrições éticas (Aguillón-Osma et al., 2019; Minekus et al., 2014). O modelo de digestão *in vitro* emprega enzimas digestivas comerciais e simula, através da associação com reagentes químicos, as condições bioquímicas presentes na fase oral, gástrica e intestinal do processo digestivo (Lee et al., 2016). Apesar de uma amplitude de estudos recentes avaliarem a bioacessibilidade de determinados compostos funcionais nos alimentos, há um número menor de informações disponíveis sobre bioacessibilidade e propriedades antioxidantes destes compostos em subprodutos vegetais, submetidos à digestão gastrointestinal *in vitro* (Gullon, Pintado, Barber, et al., 2015; Pellegrini et al., 2017).

É importante destacar que compostos que não são liberados e absorvidos no intestino delgado após a digestão gastrointestinal, podem atingir o cólon, onde podem ser metabolizados pela microbiota intestinal, promovendo efeitos biológicos distintos, como propriedades prebióticas (Cardona et al., 2013). Estas propriedades são geralmente atribuídas às frações de fibra alimentar dos subprodutos de frutas e vegetais (Diaz-Vela et al., 2013). No entanto, os polifenóis que atingem o cólon ainda ligados às fibras de matrizes vegetais complexas, podem exercer efeitos significativos na saúde intestinal, como a neutralização dos efeitos pró-oxidantes da fibra alimentar, e consequentemente promoção do crescimento da microbiota intestinal (Ana Elena Quirós-Sauceda et al., 2014). Com isso, potenciais efeitos prebióticos foram descritos em subprodutos vegetais ricos em polifenóis como subproduto de romã (Bialonska et al., 2010) e extrato e farinha de semente de uva (Cueva et al., 2013; Kwon et al., 2018).

Diante do exposto, subprodutos de frutas e vegetais podem representar fontes naturais de compostos funcionais com potencial efeito prebiótico e, avaliar o impacto da digestão gastrointestinal sobre estas propriedades, é essencial para valorizar e promover o uso de resíduos vegetais na obtenção de produtos de alto valor agregado. O objetivo deste trabalho foi avaliar o impacto da digestão gastrointestinal *in vitro* sobre o teor e perfil de carotenoides, aminoácidos e polifenóis, além da bioacessibilidade e da capacidade antioxidante da FVBP. Neste trabalho também foi avaliada a interação entre os polifenóis e a fibra alimentar da farinha após a digestão *in vitro* e, a partir dessa avaliação, determinado o potencial prebiótico.

A presente tese de doutorado está organizada em 3 capítulos que abrangem três artigos científicos que estão apresentados da seguinte forma: Capítulo I – *Bioaccessibility of Bioactive Compounds and Prebiotic Properties of Fruit and Vegetable By-products - Mini*

Review, refere-se à revisão bibliográfica aceita para publicação na revista *Current Bioactive Compounds* sobre a bioacessibilidade de compostos e propriedades prebióticas de subprodutos de frutas e hortaliças; Capítulo II – *Simulated digestion of fruit and vegetables by-product flour: amino acids, carotenoid and polyphenols stability, and changes in antioxidant capacity*, refere-se ao artigo submetido na revista *Food & Function* e aborda o impacto da digestão gastrointestinal nos compostos funcionais e capacidade antioxidante da farinha; Capítulo III – *Potential prebiotic effect of fruit and vegetable by-products flour using in vitro gastrointestinal digestion* refere-se ao artigo submetido na revista *Food Research International* que trata o potencial prebiótico da farinha de subprodutos de frutas e hortaliças, e por fim, a seção Conclusões gerais, que sistematiza as principais conclusões e perspectivas deste trabalho.

2. CAPÍTULO I - Bioaccessibility of bioactive compounds and prebiotic properties of fruit and vegetable by-products - mini review

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ABSTRACT

Background: A large proportion of the global production of fruits and vegetables is destined for processing by the food industry. This intense process generates tons of by-products, which may serve as sources of fiber and bioactive compounds, such as polyphenols and carotenoids. Accordingly, numerous studies have investigated the valorization of these by-products focusing on the extraction of bioactive compounds. However, the total amount of bioactive compounds ingested may not reflect the amount available for intestinal absorption, which refers to the bioaccessibility of these compounds. In addition, the interaction between bioactive compounds with dietary fiber and other nutrients may influence their bioaccessibility and may impair the understanding of the physiological effects of these by-products as prebiotic potential.

Methods: This mini review purpose to summarize the main results obtained in the last five years regarding the bioaccessibility of the two major bioactive compounds of fruit and vegetable by-products, i.e., polyphenols and carotenoids, to corroborate the biopotential of this food matrix. Additionally, this review attempts to elucidate the relationship between these by-products' composition and the emerging prebiotic property reported.

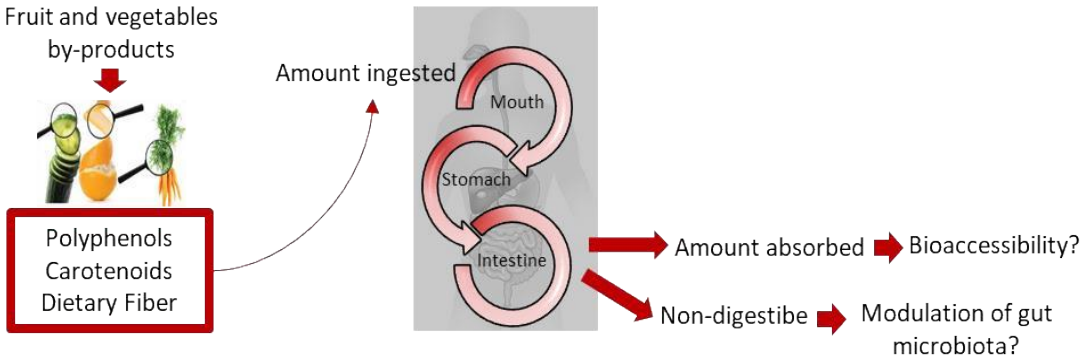
Results: In general, the bioaccessibility of polyphenols and carotenoid compounds from fruit and vegetable by-products shows high variability, and it is suggested that the composition of the food matrix is one of the main factors influencing their bioaccessibility. Moreover, a promising prebiotic effect of these by-products is described.

Conclusion: The brief literature review with recent studies provide relevant information that may contribute for using the fruit and vegetable by-products as a natural source of bioactive compounds and/ or functional ingredient.

Keywords: fruit and vegetable by-products; bioaccessibility; bioactive compounds; polyphenols; carotenoids; prebiotic properties.

Graphical Abstract

Bioaccessibility of Bioactive Compounds and Prebiotic Properties of Fruit and Vegetable By-products - Mini Review



1. Introduction

There is growing consumer demand for ready-to-eat products that combine convenience and nutritional quality with ingredients from natural sources. Consequently, the food industry is responding to increased demand for processed fruits and vegetables. This intensive processing produces a large amount of by-products, i.e., secondary products that can be consumed, but are instead discarded or redirected for non-food use (FAO, 2014).

Processed fruit and vegetable by-products include peels, seeds, leaves, stems, bagasse, and other fractions that are usually discarded. It is estimated that food processing accounts for 39% of the total food losses in Europe, with the largest contribution being from the beverage industry (26%), where generated by-products can represent up to 60% of the raw material (Amaya-Cruz et al., 2015; Baiano, 2014; European Commission, 2010). The production and discarding of these by-products represents a loss of material with high nutritional value, and signifies economic and environmental management problems (Mirabella et al., 2014).

Fruit and vegetable by-products generally have a high moisture and carbohydrate content and small amounts of protein and lipids in their composition (Mirabella et al., 2014). In addition, besides the usually high dietary fibre content, these by-products are also rich sources of bioactive compounds, mainly polyphenols and carotenoids (O'Shea et al., 2012; Padayachee et al., 2017).

Polyphenols are among the most investigated compounds, since they represent one of the largest classes of bioactive compounds and have important effects on human health (Kumar & Goel, 2019; Sagar et al., 2018). Studies with polyphenols extracts from vegetables have shown effects in the prevention of metabolic and neurodegenerative diseases, such as diabetes (Hsu et al., 2016; Mollica et al., 2017, 2018) and Alzheimer's disease (Mollica et al., 2018), through mechanisms such as the enzyme-inhibitory effect and the suppression of oxidative stress and inflammation (Hsu et al., 2016; Mollica et al., 2018).

The polyphenol content of the fruit and vegetable by-product depends on the raw material and the type of by-product (seed, shell etc.), but it is generally observed that peels and seeds have a high amount of these compounds (Sagar et al., 2018). In contrast, carotenoids are found mainly in surface tissues such as the peel and outer pericarp of fruits and vegetables (Kalt, 2005). Orange-, yellow-, and red-coloured fruits and green leafy vegetables are the main sources of carotenoid in the human diet, and their ingestion is associated with the prevention of cancer, cardiovascular disease, age-related macular degeneration, and cataract formation (Eggersdorfer & Wyss, 2018; R. K. Saini et al., 2015).

During the complex digestive process, the absorption of bioactive compounds can be influenced by the composition of the food matrix, pH, temperature, and digestive enzymes, among other factors (Bouayed et al., 2011). Thus, the amount of these compounds available for intestinal absorption may differ from the amount quantified in the non-digested food matrix (Alminger et al., 2014). In this sense, in order to evaluate the biopotential of the bioactive compounds from the food matrix, it is essential to first assess the impact of gastrointestinal digestion on their bioaccessibility. The term bioaccessibility refers to the amount of an ingested compound available for intestinal absorption after gastrointestinal digestion (A. E Quirós-Sauceda et al., 2014).

In addition to bioaccessibility, it is important to consider the interaction between the compounds in the food matrix, in order to assess whether the biological effects result from an association between the compounds (S. Liu et al., 2019). Fibre-bound polyphenols, for example, may reach the colon, where they can be metabolized by the gut microbiota, producing metabolites with distinct systemic or local effects (Cardona et al., 2013). In addition, these bound polyphenols may influence and/or contribute to the physiological properties and effects of dietary fibres (Jakobek & Matić, 2019; Ana Elena Quirós-Sauceda et al., 2014). One of these effects is the prebiotic potential.

A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). These properties are usually attributed to the dietary fibre fractions of the fruit and vegetable by-products (Diaz-Vela et al., 2013). However, by-products rich in polyphenol compounds, such as ellagitannins (Bialonska et al., 2010) and flavan-3-ol (Cueva et al., 2013; Kwon et al., 2018) have also shown potential prebiotic effects *in vitro*.

In summary, fruit and vegetable by-products may represent natural sources of bioactive compounds with potential health benefits, such as prebiotic effects (Figure 1). Thus, the purpose of this comprehensive review is to summarize the main results obtained regarding the bioaccessibility of polyphenols and carotenoids – the major classes of bioactive compounds found in fruit and vegetable by-products – in order to corroborate the biopotential of this food matrix. In addition, this review aims to elucidate the relationship between polyphenols and dietary fibre composition, given the interactions between compounds in the food matrix, and the emerging research on prebiotic properties reported for fruit and vegetable by-products.

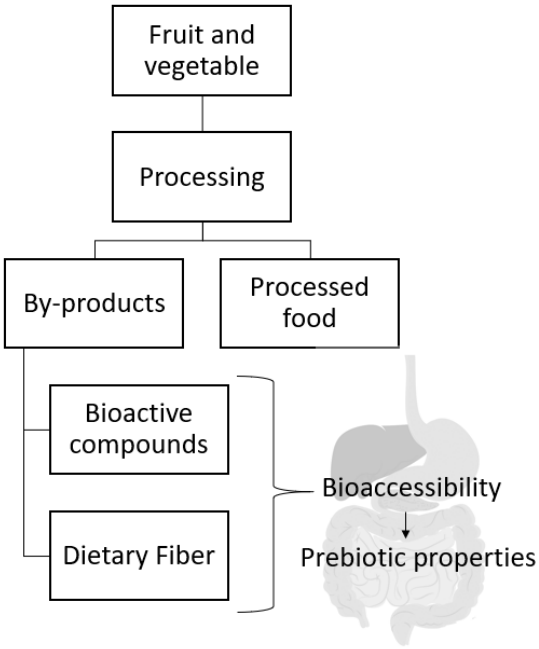


Figure **1**. Flowchart describing the process to obtain by-products from fruit and vegetable processing and its composition associated with prebiotic properties.

2. Methods for extraction of bioactive compounds from fruit and vegetable by-products

Bioactive compounds obtained from vegetable by-products can be applied in the food, pharmaceutical, cosmetic, or chemical industries (Sagar et al., 2018). However, in addition to extraction – the most critical step for obtaining bioactive compounds from plant matrix, after sample preparation –separation, identification, and characterization of these compounds is required (Azmir et al., 2013; Khoddami et al., 2013).

Different methods have been used to extract bioactive compounds and add value to plant residues (Saini et al., 2019). This extraction can be made by conventional techniques such as Soxhlet extraction, hydrodistillation, and maceration, which are based on solvent extraction, heat application, or both (Azmir et al., 2013). However, these methods have limitations such as extended extraction time, difficulty in obtaining high purity, and possible degradation of target compounds (Ajila et al., 2011; Sagar et al., 2018). Thus, non-conventional methods that have reduced extraction time, produce better yield and extract quality, and are eco-friendly, have been used as options for bioactive compound extraction (Azmir et al., 2013; Sagar et al., 2018). The non-conventional methods generally studied are enzyme-assisted extraction, pressurized liquid extraction, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and pulsed electric field-assisted extraction (Azmir et al., 2013; Saini et al., 2019).

For polyphenol extraction from a plant matrix, factors such as solvent type, time and temperature of extraction, sample matrix, and particle size may influence the extraction of these compounds (Khoddami et al., 2013). Regarding plant residue matrices, the solvent type demonstrated an effect on the quality and yield of phenolic compound extraction from guava seed (Castro-Vargas et al., 2010) and red grape pomace (Drosou et al., 2015). Due to the polarity of phenolic compounds, extraction is generally higher with high polarity solvents (Castro-Vargas et al., 2010). In addition, time and temperature parameters demonstrated influence on polyphenol extraction from pomegranate peel (Živković et al., 2018) and grapefruit solid waste (Garcia-Castello et al., 2015). Increasing time and temperature may promote solubility and consequent extraction of target compounds, however oxidation reactions or even degradation of these compounds may occur (Khoddami et al., 2013).

Non-conventional methods have been used for polyphenol extraction from plant matrices, and are generally compared to conventional methods, such as Soxhlet extraction. Supercritical fluid extraction with co-solvents, for example, showed better yield and quality of phenolic compounds from guava seeds (Castro-Vargas et al., 2010) and orange pomace (Espinosa-Pardo et al., 2017), compared to Soxhlet extraction. Other non-conventional methods demonstrating effective extraction of phenolic compounds from plant residues include ultrasound-assisted extraction in pomegranate peel (Živković et al., 2018), pulsed electric fields extraction in grape seeds (Boussetta et al., 2012), and enzyme-assisted extraction in grape residues (Gómez-García et al., 2012). In addition to the high-performance extraction of bioactive compounds, non-conventional methods have reduced processing time and reduced waste generation, which makes them green technologies (Saini et al., 2019).

Carotenoid extraction is generally performed with organic solvents, such as hexane and ethanol, due to the hydrophobicity of these compounds (Luengo et al., 2014). However, extraction with these solvents has disadvantages, such as a negative impact on the environment, in addition to the high cost of processing, and removal time for solvents such as hexane (Luengo et al., 2014; Mezzomo & Ferreira, 2016). Thus, extraction with environmentally safe, non-conventional technologies has been tested for extraction of carotenoids from plant residues. Improvement in carotenoid extraction from tomato waste was demonstrated using ultrasound under pressure (Luengo et al., 2014). In addition, supercritical fluid extraction was shown to be a viable option for carotenoid extraction from 15 different carotenoid-rich fruit and vegetable waste matrices, adding value to these by-products (Lima et al., 2019).

It is important to note that plant residues can also be used as a matrix for bio-solvent extraction applications. The bio-solvent *d-limonene*, extracted from orange processing waste, showed similar qualitative and quantitative determination in tomato lycopene extraction compared to dichloromethane solvent (Chemat-Djenni et al., 2010). In addition, combining non-conventional green technologies with bio-solvents to extract bioactive compounds from food industry by-products enhances the environmental safety of the process, and adds value to fruit and vegetable residues (Boukroufa et al., 2017).

3. Bioaccessibility of bioactive compounds from fruit and vegetable by-products

Fruit and vegetable by-products may contain more dietary fibre and phytochemical compounds, such as carotenoid and polyphenols, than the usually edible parts (Ayala-Zavala et al., 2011; O'Shea et al., 2012). However, it is important to note that the amount ingested or present in the food matrix does not reflect the amount absorbed by the body, which is then available to exert its bioactive potential (Alminger et al., 2014). After ingestion, factors such as nutrient interactions, digestive enzyme concentrations, pH, and digestion time may influence the structure and/or release of bioactive compounds from the food matrix and, consequently, the absorption and bioactivity of these compounds (Bohn et al., 2015; Cilla et al., 2018). It is estimated that only 5-10% of polyphenol compounds ingested are absorbed in the small intestine; as for carotenoid, only an estimated 10% from raw fruits and vegetables are solubilized into micelles and accessible for intestinal absorption (Cardona et al., 2013; Castenmiller & West, 1998; Estévez-Santiago et al., 2016). In addition, dietary fibre can reach the colon intact after digestion, which may influence the absorption of compounds that are associated with or entrapped in polysaccharide chains, such as polyphenols and carotenoid (Palafox-Carlos et al., 2011).

Therefore, to determine the potential beneficial effects of bioactive compounds, it is important to first assess factors such as the stability and bioaccessibility of compounds under gastrointestinal conditions (Tagliazucchi et al., 2010). *In vivo* experiments with humans are recommended to evaluate the bioaccessibility of bioactive compounds, however they are time-consuming, expensive, and carry ethical restrictions (Alminger et al., 2014). In response, simulated *in vitro* models that typically include the oral, gastric, and small and large intestinal phases have been developed to assess the release of the food matrix and bioaccessibility of bioactive compounds during gastrointestinal digestion (Alminger et al., 2014; Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carrière, Boutrou, Corredig, Dupont, Dufour, Egger, Golding, Karakaya, Kirkhus, Le Feunteun, Lesmes, MacIerzanka, et al., 2014). These

in vitro methods mimic physiological conditions *in vivo*, and take into consideration factors such as temperature, digestive enzyme action and concentration, pH and digestion time (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carrière, Boutrou, Corredig, Dupont, Dufour, Egger, Golding, Karakaya, Kirkhus, Le Feunteun, Lesmes, MacIerzanka, et al., 2014).

Although recent studies assess the bioaccessibility of bioactive compounds and nutrients in food, less information is available on the bioaccessibility of polyphenols and carotenoid from fruit and vegetable by-products after gastrointestinal digestion.

3.1. Bioaccessibility of polyphenols

Polyphenols represent a variety of substances that have similar chemical structures, with the presence of one or more aromatic rings bound to at least one hydroxyl radical and/or other substitutes. Based on their structure, polyphenol plant compounds can be categorized into several groups: mainly phenolic acids, flavonoids, lignans, and stilbenes (Spencer et al., 2008). Fruits and vegetables are rich sources of polyphenol compounds with recognized bioactive properties, including antioxidant properties that influence intracellular signalling or gene expression (Bouayed et al., 2012).

Research studies on the bioaccessibility of polyphenols from fruit and vegetable by-products indicate change in total compounds available for absorption after digestion. The polyphenol bioaccessibility of date pits and apple bagasse flour after digestion was 78.54% and 91.58%, respectively (Gullon, Pintado, Barber, et al., 2015); for *Moringa oleifera* seed flour,78.42% and 56.20% of bound and free polyphenols, respectively, were bioaccessible after digestion (Swetha et al., 2018). Similarly, the mean phenolic compound bioaccessibility of six quinoa seeds was about 73% after digestion; however, flavonoid bioaccessibility was only about 13% (Pellegrini et al., 2017). In contrast, pomegranate peel flour showed phenolic and flavonoid bioaccessibility after digestion of 35.90% and 64.02%, respectively (Gullon, Pintado, Fernández-López, et al., 2015). For the cashew apple fibre solid by-product, bioaccessibility of extractable polyphenols after *in vitro* digestion was 18.6% (Lima et al., 2014). Additionally, bioaccessibility was about 50% for phenolics, and between 21.54% and 45.31% for flavonoids in persimmon fruit co-product flours, depending on the persimmon cultivar (Lucas-González et al., 2018).

It has been observed that polyphenols, released from fruit and vegetable by-products during digestion, can be highly influenced by the composition of the food matrix, particularly by constituents such as fibre and protein (Gullon, Pintado, Fernández-López, et al., 2015;

Lucas-González et al., 2018; Ortega et al., 2011; Pellegrini et al., 2017). For example, the interaction of polyphenols with dietary fibre, and their complex formation with matrix minerals, may reduce solubility and influence bioaccessibility (Lima et al., 2014). In contrast, polyphenol interaction with the soluble fraction of the food matrix, such as sugar and soluble fibre, may exert a protective effect on polyphenol stability during digestion, thereby improving bioaccessibility (Ortega et al., 2011). Thus, it is suggested that food matrix composition is one of the main factors influencing the bioaccessibility of polyphenols from by-products. In addition, the chemical diversity of these compounds, which can range from simple to highly polarized molecules, may influence their release from the food matrix, different pH-dependent transformations, and interactions with other food components (Alminger et al., 2014; Schulz et al., 2017).

It is important to note the effect of processing on the bioaccessibility of bioactive compounds. Cilla et al. (2018b) concluded that, in general, thermal treatment and ultrasound processing can improve the bioaccessibility of food polyphenols. During processing,the rupture and transformation of the natural matrix can influence the release of compounds, increasing the possibility of absorption in the digestive tract (Parada & Aguilera, 2007). In addition, studies assessing the effect of processing on polyphenol bioaccessibility using the whole matrix of fruit and vegetable by-products, versus polyphenol extract alone, are still scarce.

Concerning the effect of gastrointestinal digestion phases on the stability of polyphenols from fruit and vegetable by-products, it has been observed that the oral phase is less effective for the release of these compounds from food matrix. Similarly, for *Moringa oleifera* seed flour, bioaccessibility of phenolic compounds at the oral phase was lower, compared to other phases of digestion for most analysed compounds (Swetha et al., 2018). The minimal change at this phase was attributed to the short exposure time and the marginal effects of α-amylase (Mosele et al., 2016). It has been suggested that there is an increase in polyphenol release during the gastric phase, likely due to hydrolysis, induced by acidic pH and protease activity (Rodríguez-Roque et al., 2013; Saura-Calixto et al., 2007). The increased polyphenol compound content and bioaccessibility after the gastric phase was found for the co-products of pomegranate peel flour (Gullon, Pintado, Fernández-López, et al., 2015) and persimmon fruit flour (Lucas-González et al., 2018).

After the intestinal phase, variation in the polyphenol content of the food matrix is generally observed (Gullon, Pintado, Barber, et al., 2015; Gullon, Pintado, Fernández-López,

et al., 2015; Lucas-González et al., 2018). This can be explained by interaction with other dietary compounds, changes in molecular structure and solubility, and chemical reactions like oxidation and polymerization (Gullon, Pintado, Fernández-López, et al., 2015; Lucas-González et al., 2018). In contrast, since polyphenols represent a chemodiverse group, the increased release of this compound from the by-product matrix at the intestinal phase was also described (Pellegrini et al., 2017; Swetha et al., 2018). This release is probably associated with the alkaline media at this phase, which may favour the extraction of free and bound polyphenols (Swetha et al., 2018). In addition, two factors related to the intestinal phase environment may affect the release and, consequently, the bioaccessibility of polyphenols:

1. Through its amylase and protease activity on the food matrix, the pancreatin enzyme may improve the release of nutrient-bound polyphenols in the food matrix (Bouayed et al., 2011); and
2. the presence of bile acids that can bind to dietary fibre, which reduces polyphenol entrapment, may release these compounds from the food matrix at the intestinal phase (Yang et al., 2018).

Another important factor to consider when assessing the bioaccessibility of polyphenols are the compounds strongly linked to the food matrix. After gastrointestinal digestion, these compounds may reach the colon, where they can be fermented by the gut microbiota, producing absorbable metabolites and contributing to the reported health effects of recognized polyphenols (Pérez-Jiménez et al., 2013). It is estimated that, depending on the type of dietary intake, 48% of polyphenols from solid vegetables are bioaccessible in the small intestine and 42% in the large intestine, either in their original structure, or as fermentation metabolites (Saura-Calixto et al., 2007).

3.2. Bioaccessibility of carotenoid

Carotenoids are lipophilic compounds classified into two types, according to their functional groups: xanthophylls, such as lutein and zeaxanthin, with oxygen as a functional group; and carotenes, such as α- and β-carotene and lycopene, with a pure hydrocarbon chain (R. K. Saini et al., 2015). Regular intake of carotenoid is associated with various beneficial health effects, such as the reduced risk of developing cancer, cardiovascular disease, cataracts, and macular degeneration (Krinsky & Johnson, 2005; Rodriguez-Amaya, 2018). These effects are mainly associated with carotenoids'antioxidant properties; however, other mechanisms have been investigated, such as growth inhibition in tumour cell lines, antimutagenic action, and modification of cell-cell communication (Krinsky & Johnson, 2005).

Fruit and vegetable by-products may represent a significant source of carotenoids (Abdul Aziz et al., 2012; Silva et al., 2014; Wang et al., 2010). However, bioavailability for normal metabolic processes depends on initial bioaccessibility for intestinal absorption (Palafox-Carlos et al., 2011). This term is related to the amount of carotenoid that is released from the food matrix and incorporated into micelles after gastrointestinal digestion, thereby becoming available for gut absorption (Rodriguez-Amaya, 2015).

Several factors may influence carotenoid bioavailability, including the described by the mnemonic SLAMENGHI: Species of carotenoids, molecular linkage, amount of carotenoids consumed, matrix, effectors of absorption and bioconversion, nutrient status of the host, genetic, host-related factors, and mathematical interactions (Castenmiller & West, 1998). The main factors that influence bioaccessibility of carotenoids from fruits and vegetables are the food matrix, the effectors for absorption, interaction with other nutrients, and previous food processing (Barba et al., 2017; Kopec & Failla, 2018; Lemmens et al., 2014; Xavier & Mercadante, 2019).

It is well recognized that carotenoid from fruit and vegetables have low bioaccessibility, which varies according to carotenoid structure (Estévez-Santiago et al., 2016; Kaulmann et al., 2016). This low bioaccessibility can be explained by the low lipid and high fibre content of fruit and vegetable matrices. The noncovalent bond of proteins and fibres to the matrix, and the entrapment of bile acids and lipids by dietary fibre, may reduce the bioaccessibility of carotenoid (Palafox-Carlos et al., 2011; Parada & Aguilera, 2007). In contrast, dietary lipids play an important role in the absorption of these compounds by stimulating bile acid secretion into the intestine, causing the carotenoid to solubilize into micelles (Palafox-Carlos et al., 2011; Parada & Aguilera, 2007). Different carotenoids can vary significantly by their structure. Carotenes, such as β-carotene and lycopene, have a pure hydrocarbon chain, so arefat-soluble; by contrast xanthophylls, such as the more polar lutein and zeaxanthin, contain oxygen in their chains (Krinsky & Johnson, 2005).

Despite the growing interest in fruit and vegetable by-products as alternative sources of bioactive compounds and functional ingredients, studies assessing the carotenoid bioaccessibility of these by-products are still scarce. In a recent study, an Ataulfo mango peel demonstrated high β-cryptoxanthin bioaccessibility (around 35%), and around 20% β-carotene and lutein bioaccessibility (Mercado-Mercado et al., 2018). In the same study, the Ataulfo mango paste by-product showed no statistical difference in bioaccessibility of β-cryptoxanthin, β-carotene and lutein; all were around 30-40% (Mercado-Mercado et al.,

2018). In contrast, another study showed that the bioaccessibility of β-carotene in Ataulfo mango puree was about 6%, with no assessed difference between ripening stages (Schweiggert et al., 2012). It is important to note that there are differences in the analytical methods employed by the cited studies. The bioaccessibility of fruit and vegetable by-products is not totally understood, and it may differ in comparison with usually edible parts. Therefore, further studies are needed to confirm these differences.

In relation to the influence of the food matrix, it has been suggested that the dietary fibre content of fruit and vegetable by-products, which is usually high, is a limiting factor for carotenoid bioaccessibility in this matrix. Dietary fibre can increase the viscosity of food digests and entrap or bind to lipophilic compounds and bile acids, which influences micelle formation and decreases the bioaccessibility of lipophilic compounds (M. Tomas et al., 2018). A study of eight leafy vegetables consumed in Southeast Asia demonstrated that carotenoid bioaccessibility was negatively correlated to the pectin content of the leaves (Sriwichai et al., 2016). The gel-like pectin formed in gastrointestinal conditions may increase the viscosity in the duodenal medium, which impairs the activity of lipase and bile acid effects, while inhibiting micelle production (Sriwichai et al., 2016).

The by-products obtained from fruit and vegetable processing, however, may increase carotenoid bioaccessibility. The mechanical or thermal processing methods that disrupt the natural barrier of the plant cell wall and adjacent cell clusters may increase carotenoid release during digestion and promote bioaccessibility (Kopec & Failla, 2018). Carotenoids found in dark green leafy vegetables, for example, may be entrapped or in a complex with proteins in chloroplasts and within cell structures (Castenmiller & West, 1998). Thus, the higher lutein and β-carotene bioaccessibility of processed, pureed spinach, compared to whole leaf, can be attributed to the mechanical disruption of the food matrix (Eriksen et al., 2017). Similarly, microwave and steam processing pre-treatment increases the β-carotene bioaccessibility of sweet potato flour (Trancoso-Reyes et al., 2016).

Carotenoid bioaccessibility can also be influenced by gastrointestinal digestion steps. The peel and paste by-products of the Ataulfo mango show higher β-cryptoxanthin, β-carotene, and lutein content in the intestinal versus the gastric fraction of *in vitro* digestion (Mercado-Mercado et al., 2018). Studies with fruit and vegetable pulp show differences in carotenoid stability during *in vitro* digestion, with losses occurring during the digestive phase (Petry & Mercadante, 2017) or increasing after the intestinal phase (Hedrén et al., 2002). In addition, after digestion, non-absorbed carotenoids can reach the colon, but metabolite

production at this phase and its relationship with the intestinal microbiota is not well described in the literature (Xavier & Mercadante, 2019). Thus, further studies are needed to understand the stability and bioaccessibility of carotenoids from fruit and vegetable by-product sources during and after gastrointestinal digestion.

4. Prebiotic properties of fruit and vegetable by-products

Most prebiotic studies have focused on the selective stimulation of *Bifidobacterium* and *Lactobacillus* growth (Roberfroid et al., 2010; Watson et al., 2013). However, the healthy human gut microbiota consists of hundreds of species, 98% of which belong to the phylum Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Falony et al., 2009). The Firmicutes and Bacteroidetes phyla are the most abundant, with a prevalence of clostridial clusters IV and XIV, and *Bacteroides* and *Prevotella* genera, respectively (Graf et al., 2015; Louis et al., 2007). Other less abundant, but prevalent phylum are Actinobacteria, which includes the genera *Bifidobacterium*, Proteobacteria, which includes *Escherichia coli*, Fusobacteria, with genera *Fusobacterium*, and Verrucomicrobia, including the *Akkermansia* genera (Bik et al., 2018; Graf et al., 2015). It is important to note that *Lactobacillus* (of the Firmicutes phylum) and *Bifidobacterium* usually represent less than 5% of the total healthy human gut microbiota (Louis et al., 2007).

The selective effect of a prebiotic substrate may extend to various microbial groups, and it should induce a health benefit, such as the production of metabolites like short chain fatty acids (SCFA) (Gibson et al., 2017). SCFA, such as acetate, propionate, and butyrate, are linked to intestinal epithelial energy metabolism, inflammation suppression, anti-carcinogenic effects, and appetite suppression regulating effects (Frost et al., 2014; Louis et al., 2014).

Polysaccharides from fruits and vegetables, like cellulose, hemicellulose, and pectin, generally represent the greater part of by-products such as peels, seeds, and bagasse polymer composition (de la Rosa et al., 2019; Yoo et al., 2012). These polysaccharides and/or their non-digestible hydrolysed oligomers may reach the colon intact, where they may be useful as nutrients for gut microbiota fermentation and stimulation of SCFA production. Thus, recent studies have investigated the potential prebiotic effects of whole fruit and vegetable byproducts (Table 1), and their possible role as a natural, sustainable, and cost-effective prebiotic source (Gullón et al., 2009; Reichardt et al., 2018; Vazquez-Olivo et al., 2019).

Table **1**. Summary of the main studies with by-products of fruits and vegetables with prebiotic potential assessed.

By-product	Fruit/Vegetable	Model of investigation	Prebiotic effect	Reference
Mashed peel	Cashew apple	Microplate assay	Positive prebiotic activity scores in *Lactobacillus* strains, pH decrease, organic acid production and sugar consumption over 48h.	(Duarte et al., 2017)
Peel	Mango	In vitro model of the proximal colon (TIM-2) using human fecal microbiota	Increased abundance of *Bifidobacterium* strains within 24 hours of fermentation and increasing short chain fatty acid production.	(Sáyago-Ayerdi et al., 2019)
Seed	Grape	*In vitro* fermentation assay – Standard plate count	Stimulation of probiotics *Lactococcus lactics* subsp. *Lactics*, *Leuconostocmesenteroide*, *Lactobacillus kefiri* DH5 bacteria and inhibition of pathogenic bacteria *Clostridium perfringes*.	(Kwon et al., 2018)
Albedo; peel Peel	Grapefruit Cactus pear Pineapple	*In vitro* fermentation assay – Standard plate count	Fermentable carbon source by lactic acid bacteria (*P. pentosaceus*UAM21 and *A. viridans* UAM22) with an acceptable short chain organic acids production.	(Parra-Matadamas et al., 2015)
Peel	Cactus pear Pineapple	*In vitro* fermentation assay – Standard plate count	Fermentable carbon source by lactic acid bacteria (*Pediococcuspentosaceus* UAM22, *Aerococcusviridans*UAM21 and *Lactobacillus rhamnosus* GG) with higher organic acid production compared with glucose.	(Diaz-Vela et al., 2013)
Peel; seed Peel	Orange Acerola Passion fruit Mango	*In vitro* fermentation assay – Standard plate count	Acerola by-product showed the highest selectivity for beneficial bacteria (seven *Lactobacillus* spp. And three *Bifidobacterium* spp. Strains). Acerola, mango, and orange by-products have the greatest potential to be used as prebiotic ingredients.	(Vieira et al., 2017)

Table **1**. (Continued)

Bagasse Peel	Orange Passion fruit	*In vitro* model of the proximal colon (TIM-2)	*Bacteroides* and *Ruminococcus* were the main genera stimulated by insoluble fraction of fruit by-products. Fermentation of Orange bagasse with more soluble material generated similar amounts of total SCFA as inulin (positive control).	(De Souza et al., 2019)
By-product (extract)	Pomegranate	Batch-culture fermentation with human fecal microbiota	Pomegranate by-product extract enhanced the growth of total bacteria, *Bifidobacterium* spp. And *Lactobacillus* spp., without influencing the *Clostridium coccoides–Eubacteriumrectale group* and the *C. histolyticum* group. In addition, increased concentrations of short chain fatty acids (SCFA).	(Bialonska et al., 2010)
Seed (extract)	Grape	Microplate assay (Growth curves) *In vitro* fermentation assay – Standard plate count	Increased the growth of *Lactobacillus* and *Bifidobacterium* strains with production of acetic, butyric, formic, and propionic acids during fermentation.	(Costa et al., 2019)
Peel	Passion fruit	Cecal microbiota of Male Wistar rats	Increased in SCFAs intestinal production by Wister rats, however, not changed colonic microbiota counts.	(Silva et al., 2014)

A promising prebiotic effect from fruit and vegetable by-products is observed, but in studies utilizing the whole matrix of these by-products, the association between effect and matrix composition is unclear. Therefore, the differences in food matrix composition and digestibility should be considered in order to assess potential prebiotic effects. However, recent studies have focused on the extraction of non-digestible oligosaccharides derived from complex polysaccharides, mainly pectin, and on xylans (Yoo et al., 2012), such as pectic oligosaccharides (Gómez et al., 2019) and xylooligosaccharides (XOS) (Kaur et al., 2018).

It is important to highlight that polysaccharide-bound polyphenols can be released into the colon by gut microbiota fermentation and converted to metabolites with distinct health effects, and can also act on gut microbiota modulation (Saura-Calixto, 2011; Tomás-Barberán et al., 2016). It has been suggested that a whole matrix of fruit and vegetable by-product substrates may exert a positive effect on healthy microbiota growth, possibly resulting from the shared action of oligo- and polysaccharides, and polyphenols and their derived metabolites. In agreement, Liu et al. (2019) demonstrated that bound polyphenols contributed significantly to the prebiotic properties of dietary fibre from carrots (S. Liu et al., 2019). Moreover, Bordiga et al. (2019) demonstrated the prebiotic activity of oligosaccharide fractions extracted from grape seed (Bordiga et al., 2019). However, they previously performed polyphenol extraction from the sample, since they considered this factor as a variable that may influence the result. Similarly, Sáyago-Ayerdi et al. (2019a) attributed the assessed prebiotic potential of mango peel to their indigestible fraction, comprised of soluble and insoluble fibres, polyphenols, resistant protein, and other non-digestible compounds (Sáyago-Ayerdi et al., 2019).

In addition, Bialonska et al. (2010) reported the largest increase in growth of faecal microbiota with pomegranate by-product extract, compared to isolated punicalagins extracted from pomegranate alone (Bialonska et al., 2010). The authors attribute this difference to the presence of oligomers composed of gallic acid, ellagic acid, and glucose in different combinations in the pomegranate by-product extract. Additionally, a grape pomace extract rich in non-digestible fibre, and with significant amounts of extractable gallic and ellagic acids, showed a tendency to promote the growth of the gut microbiota, but with significant results only for the *Enterococcus* group (Gil-Sánchez et al., 2017). However, in studies with *in vitro* gastrointestinal digestion models, grape pomace extract showed potential to promote modulation of gut microbiota, with higher growth of *Lactobacillus* and *Bacteroides* groups (Gil-Sánchez et al., 2018). These studies show a potential prebiotic effect of fruit and

vegetable by-products and compounds extracted from this matrix; however, it is observed that interactions with certain compounds may influence bacterial growth.

Further studies are needed to investigate the structure and interaction of compounds in the whole food by-product matrix before and after gastrointestinal digestion, and to explore the impact on the modulation of gut microbiota. Such studies may extend the use of by-products as functional food ingredients, and as a source of functional compounds.

5. Recent trends in fruit and vegetable by-product use

The treatment, characterization, and incorporation of agro-industrial by-products in foodstuffs reintroduces these by-products into the food chain and reduces waste, a trend in the circular economy model (Castro-Muñoz et al., 2018; Trigo et al., 2019). In addition to environmental and economic advantages, the addition of agro-industrial by-products may improve the nutritional characteristics of newly-developed foods, due to their dietary fibre- and bioactive compound-rich compositions (Bhol et al., 2016; Toledo et al., 2019), and their potential contribution to positive health effects such as reduced cardiovascular risk (Mildner-Szkudlarz & Bajerska, 2013).

Supplementation with agro-industrial by-products has shown protective effects in protecting colon and liver tissue from damage induced by a dyslipidaemic diet (Batista et al., 2018) and in preventing the development of fatty liver disease and hyperglycaemia (Amaya-Cruz et al., 2015). In other fields, the effects of agro-industrial by-product use have recently been described, including the antifungal effect against Candida and dermatophytes, and the biostimulant effect (Abou Chehade et al., 2018; Sánchez-Gómez et al., 2017), which may represent an option for use in place of chemical fertilizers.

6. Conclusion

Fruit and vegetable by-products can be considered a natural source of bioactive compounds and the health-related effects of these compounds have been widely studied. Thus, different techniques of extraction of bioactive compounds were developed, mainly to obtain bioactive compounds from natural sources. However, the effect of extracts of bioactive compounds are generally evaluated without considering the bioaccessibility of these compounds, including interaction with other substances or nutrients.

In general, the bioaccessibility of phenolic and carotenoid compounds from fruit and vegetable by-products shows high variability. It is suggested that the composition of the food matrix is one of the main factors influencing the bioaccessibility of polyphenols from fruit and vegetable by-products. However, factors such as the chemodiversity of these compounds

and the previous processing of the food matrix are also important. Similarly, the bioaccessibility of carotenoids can be influenced mainly by their structure, the food matrix composition, and previous processing.

In addition, it is observed a promising prebiotic effect from fruit and vegetable by-products, but it is suggested that, with this whole matrix as substrate, the potential prebiotic effect may be a result of the shared action of compounds, mainly prebiotic oligo- and polysaccharides and available polyphenols and metabolites. However, other nutrients as protein and peptides also may influence on this effect. Thus, further studies are needed for assess the effect of each compound's structure and their interaction on the modulation of gut microbiota.

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3. CAPÍTULO II – Simulated digestion of fruit and vegetables by-product flour: amino acids, carotenoid and polyphenols stability, and changes in antioxidant capacity

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ABSTRACT

The present work aimed to evaluate the changes in amino acids, carotenoid and polyphenols content, and antioxidant capacity of fruit and vegetable byproduct flour (FVBP flour) during *in vitro* gastrointestinal digestion. The total polyphenols content (TPC) and profile, carotenoid and free amino acids profile and antioxidant capacity of FVBP flour were evaluated at initial, oral, gastric and intestine digestion phases. The TPC and antioxidant capacity were also determinate in soluble and pellet fractions of FVBP flour. In addition, the profile, bioacessibility and recovery index of polyphenols from FVBP flour and bound polyphenol content (BPC) to insoluble fiber fraction was also assessed. After *in vitro* digestion, the recovery index of TPC decreased associated with a relevant decrease of recovery of TPC in soluble fraction and an increase in pellet fraction. Consequently, lower value of polyphenol bioaccessibility of FVBP flour was obtained, of 37.7%. However, the antioxidant capacity and total content of flavonoids showed an increase after intestine phase. The β-carotene content showed no variation and lutein and zeaxanthin content increased after *in vitro* digestion. An increase in all amino acids content was obtained, with higher amounts of glutamine and arginine after intestine phase. These findings suggest that there is an increase in carotenoid, amino acid and flavonoids contents after digestion, as well as an increase in antioxidant capacity. Thus, FVBP flour can be used as a source of bioactive compounds and as functional ingredient, contributing to improve the value of foodstuffs.

Keywords: fruits and vegetables by-products; functional flour; bioactive compounds; amino acids; antioxidant capacity; *in vitro* digestion.

1. Introduction

Processing fruits and vegetables generates tons of by-products including shells, stems, seeds, bagasse, pulp among other parts, which can represent up to 60% of processed raw material depending on the raw material and the applied process (Amaya-Cruz et al., 2015). These by-products are potential sources of bioactive compounds such as polyphenols (Ayala-Zavala et al., 2011; Gullon, Pintado, Fernández-López, et al., 2015), carotenoid (Goula, Ververi, Adamopoulou, & Kaderides, 2017; Wang, Chuang, & Hsu, 2008), dietary fiber (Kowalska et al., 2017; Pérez-Jiménez & Viuda-Martos, 2015) and nutrients such as amino acids (Egydio et al., 2013; Rohsius et al., 2006). All of these compounds have been associated with health effects that may be influenced by factors such as the amount ingested and the bioaccessibility of the compound (Bouayed et al., 2012).

Several studies demonstrate that bioactive compounds and nutrients can be totally or partially released from the food matrix during gastrointestinal digestion and-/or be biotransformed into compounds with different biological activity (Bouayed et al., 2012; Cilla et al., 2018; Kaulmann et al., 2016; Tagliazucchi et al., 2010). The variation in this release from food matrix is related to factors such as compound solubility, interactions with macromolecules and the biochemical extraction conditions, such as pH. Dietary fiber, for example, can affect the release of carotenoids and polyphenols compounds by the entrapment of these compounds during gastrointestinal digestion (Palafox-Carlos et al., 2011). Thus, assessing the nutrient bioaccessibility associated with a food matrix, and not isolated or as an extract, is the first step to determine its potential bioactive.

Bioaccessibility can be defined as the content of compound released from food matrix during gastrointestinal digestion and that is available for absorption into the intestinal mucosa (Saura-Calixto et al., 2007). The *in vitro* gastrointestinal digestion models have been widely used to evaluate the effects of digestion on bioaccessibility of nutrients since they are fast, less expensive and with no ethical restriction, compared to *in vivo* studies (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carrière, Boutrou, Corredig, Dupont, Dufour, Egger, Golding, Karakaya, Kirkhus, Le Feunteun, Lesmes, MacIerzanka, et al., 2014). These models simulate physiological *in vivo* conditions using commercial digestive enzymes and chemical reagents for oral, gastric and intestinal (small intestine) digestion phases and occasionally intestinal fermentation (large intestine) (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carrière, Boutrou, Corredig, Dupont, Dufour, Egger, Golding, Karakaya, Kirkhus, Le Feunteun, Lesmes, MacIerzanka, et al., 2014; Saura-Calixto et al., 2007).

Macronutrients are generally digested efficiently after the digestive process (Kopf-Bolanz et al., 2012). However, molecular interactions as occur between protein and polyphenols compounds from vegetable matrix, can affect nutrient digestibility during gastrointestinal digestion (Dufour et al., 2018). It is important to note that changes in protein digestibility can affect their availability in the diet and, consequently, the supply of amino acids and nitrogen to meet metabolic needs (FAO, 2011). In this sense, in vegetable sources, the assess of protein digestibility and molecular interactions in the digestive process is essential since, in general, these sources have already reduced content of essential amino acids when compared to animal protein sources (Mathai et al., 2017; Tessari et al., 2016). The essential amino acids are not synthesized by the organism and depend on nutrition to supply the metabolic need (Tessari et al., 2016), which highlights the need to assess protein composition during gastrointestinal digestion.

Despite the impact of food matrix interactions on bioacessibility of bioactive compounds and nutrients, studies food products resulting from agro-industrial fiber-rich by-products submitted to gastrointestinal digestion are scarce (Gullon, Pintado, Barber, et al., 2015). Thus, the aim of the present work was to evaluate the content and profile of polyphenols, carotenoids and free amino acids, as well as antioxidant capacity of a vegetable flour produced from byproducts of fruit and vegetable processing, before and after gastrointestinal digestion using an *in vitro* digestion model. In addition, the association of polyphenols with the insoluble dietary fiber fraction during *in vitro* digestion was also evaluated.

2. Material and methods

2.1. Sample

The fruit and vegetables by-products flour (FVBP flour) was obtained from the remaining solid byproduct of the processing of eleven species of fruit and vegetables, according to Ferreira et al. (2015). This flour presents a high dietary fiber content (48%), with higher insoluble fiber fraction content (39%) than soluble fraction (9.6%) and a significant content of available carbohydrates (26%) and proteins (9.5%) (Roberta M.S. Andrade et al., 2016; Ferreira et al., 2015). In addition, the FVBP flour show an average of particle size of 350 μm FVBP flour (Andrade et al., 2016).

2.2. *In vitro* gastrointestinal digestion

FVBP flour was submitted to simulated oral, gastric and intestinal steps according to the method described by (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carrière, Boutrou, Corredig, Dupont, Dufour, Egger, Golding, Karakaya, Kirkhus, Le Feunteun,

Lesmes, MacIerzanka, et al., 2014), with few modifications (Table 1). Initially, 22.5 g of the sample were suspended in 150 mL of tap water and pH value was adjusted to 6.0 ± 0.02 using HCl (1 M).

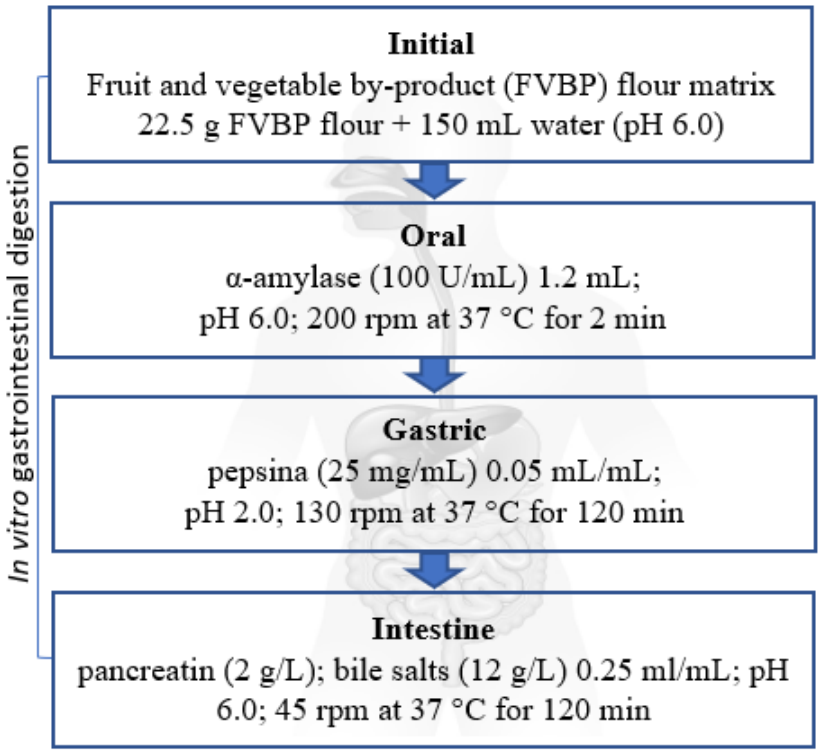

Figure 1. Scheme of *in vitro* gastrointestinal digestion procedure according to Madureira et al. (2011), with modifications.

The oral step was simulated by the addition of 1.2 mL of α-amylase (100 U/mL) (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) to the suspension and incubation for 2 minutes at 37 °C and 200 rpm in a shaking water bath (Julabo GmbH, Seelbach, Germany). For the gastric step, the pH value solution was adjusted to 2.0 with HCl (1 M), and pepsin (25 mg/mL) (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) was added at the ratio of 0.05 mL/mL of solution. This solution was incubated for 120 minutes in a shaking water bath at 37 °C and 130 rpm. After, for intestinal step, the pH value was adjusted to 6.0 using $NaHCO_3$ (1 M) and a mixture of pancreatin (2g/L) (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) and bile salts (12 g/L) (Oxoid ™, Hampshire, UK) was added at a ratio of 0.25 ml/ml of solution. This solution was incubated for 120 minutes in a shaking water bath at 37 °C and 45 rpm. After each gastrointestinal digestion phase (initially, oral, gastric and intestinal), aliquots

of approximately 5 g were collected and freeze-dried (Christ freeze dryer Alpha 1-4, Osterode Am Harz, Germany), and then stored in desiccator with silica at room temperature for further analysis.

2.3. Free amino acid and carotenoid profile and content

2.3.1. Free amino acids profile and content

The free amino acids profile of each digestion freeze-dried aliquots was determined as described by Pripis-Nicolau, De Revel, Bertrand & Maujean (2000), by Liquid chromatograph with High Resolution Fluorescence Detector and "autosampler". Initially, freeze-dried samples (50 mg) were extracted ultrasonically according to Caidan et al. (2014) with acetonitrile-methanol (1: 1, v: v) for 30 minutes. The samples were then centrifuged for 15 minutes and 4000 g at room temperature and the collected extract was filtered through filter paper.

The Chromolith® Performance RP18 (4.6 x 100 mm) column (Merck (REF: 1.02129.0001) was used and the chromatographic conditions were as follows: flow rate: 0.8 mL / min; detector: λ excitation 356 nm, λ emission 445 nm. The derivatization reaction was performed by the Spark Midas auto-sampler: To 100 µL of sample, were add 250 µL of reagent A (3 mL of Internal Stardard with homoserine and norvaline (20 mg/L) + 120 µL of mercaptoethanol + 500 mg of sodium tetraphenylborate, complete to 25 mL with borate buffer), and 250 µL of reagent B (3.5 g of iodoacetic acid with 50 mL of borate buffer, pH 9.5). The mixture was homogenized and after 3 minutes 250 µL of reagent C was added (225 mg of OPA (o-phthaldialdehyde) + 5 mL of methanol + 50 mL with borate buffer + 0.5 mL of mercaptoethanol). The mixture was homogenized again and after 3.5 minutes the injection was made. Separation and quantification were performed by injecting 10 µL of derivate using a HPLC system (Waters 410 separation module and 474scanningfluorescencedetector). The total content of each amino acid assessed was expressed in mg / g of dry raw sample.

2.3.2. Carotenoid profile and content

The total carotenoid content was extracted from freeze-dried samples of all digestion steps and carried out as described by Wright & Kader (1997). Initially, approximately 0.5 g of the sample was diluted in ethanol and homogenized in Ultra-Turrax for 3 minutes and 10.000 rpm. After, approximately 8 mL of hexane were added, and the sample homogenized for 2 minutes more. The resulting mixture was centrifuged at 4000 x g for 10 minutes and the supernatant hexane layer was then collected. In addition, 5 mL of saturated sodium chloride solution and 8 mL of hexane were added to the solid residue of centrifugation for a second

extraction. The mixture was homogenized in Ultra-Turrax for 1 minute. Thereafter, the collected supernatant hexane layers were used for saponification analysis.

For saponification, 10% methanolic KOH was added to the hexane layer and the mixture kept stirring overnight (16 h) at 300 rpm. The mixture was then washed with 10% NaCl solution and the resulting solution containing the carotenoids was used for analysis of carotenoids profile and content (Kimura et al., 1990; Oliveira et al., 2014). The identification and quantification of carotenoids profile of each digestion step aliquot was determined by HPLC-DAD, as described by Oliveira et al. (2014). The chromatographic conditions used were as follows: Flow rate: 1.0 mL/min; Column temperature: 25 ° C; Time: 20 minutes; Injection volume, 40 μL; Detection wavelength: 454 nm; Reverse phase Symmetry® C18 column (250 mm × 4.6 mm i.d., particle size 5 μm and 125 A pore size); Guard column containing the same stationary phase (Simmetry® C18). Carotenoids were eluted using the ratio 55:22:11.5:11.5:0.02 (v/v/v/v/m) of acetonitrile, methanol, dichloromethane, hexane and ammonia acetate, respectively, under isocratic conditions. The β-carotene, zeaxanthin and lutein were used as reference standards and a calibration curve of each standard was calculated with different concentrations. The result was determined by the mean of three replicates.

2.4. Total (extractable) and bound (non-extractable) polyphenol content, profile, recovery index and bioaccessibility of polyphenols

2.4.1. Sample preparation

For analysis of total and bound polyphenol content, recovery and bioaccessibility, the aliquots of each gastrointestinal digestion phase were centrifuged at 4 °C and 4000 g for 15 minutes to separate the supernatant (Soluble fraction - SF) and pellet (Pellet fraction - PF). This both fractions were freeze-dried and stored at same conditions referred above.

The extraction of polyphenols compounds from the FVBP flour and their PF and SF was made using 100% distilled water as solvent, according to Santos & Gonçalves (2016), with modifications. All freeze-dried samples of digestion steps were diluted in distilled water to a concentration of 6% (w / v) and homogenized by Ultra- Turrax (T 18 Digital Ultra-Turrax, Wilmington, USA) for 3 minutes at 8000 rpm. After, the samples were shaken at 40 °C and 200 rpm for 4 h in incubated shaker (Wiggenhauser, Berlim, Germany). The samples were then centrifuged for 15 minutes and 5000 rpm at room temperature. The collected extract was filtered through filter paper and used for the analysis of total and bound polyphenols content.

2.4.2. Total polyphenol content (Extractable polyphenols)

The total polyphenol content (TPC) was performed with FVBP flour and their SF and PF aqueous extract of all digestion phases by the Folin Ciocalteu's method (Singleton & Rossi, 1965), using gallic acid as standard reference. Each sample was analyzed in triplicate and the result expressed in mg gallic acid equivalent (eq.) /g sample.

2.4.3. Recovery index and Bioaccessibility index of polyphenols

The recovery and bioaccessibility index of polyphenols were determined according to Ortega et al. (2011). The percentage of recovery was calculated by the total polyphenols content (TPC) of FVBP flour after each phase of *in vitro* digestion and the content present in FVBP matrix before digestion. This index reflects the percentage of polyphenols compounds recovery after each digestion phase and was calculated as follows:

$$\text{Recovery index (\%)} = \frac{\text{TPC (DS)}}{\text{TPC (FVBP)}} \times 100 \qquad \text{(Eq. 1)}$$

where TPC (DS) is the total polyphenol content (mg) of each phase of FVBP digested sample and TPC (FVBP) is the total polyphenol content (mg) quantified in 1g of undigested FVBP flour (SF + PF).

The bioaccessibility index was calculated by comparing the content of polyphenols present in the soluble fraction (SF) with the content of FVBP (PF + SF) after the intestine step, according to Ortega et al. (2011), as follows:

$$\text{Bioaccessibility index (\%)} = \frac{\text{TPC (sf)}}{\text{TPC (df)}} \times 100 \qquad \text{(Eq. 2)}$$

where TPC (SF) is the total polyphenol content (mg) in the SF after the intestine step and TPC (DS) is the total polyphenol content (mg) in the FVBP flour (SF + PF) after the intestine step.

2.4.4. Bound polyphenol content (BPC) (Non-extractable polyphenols) from insoluble fraction

Initially, insoluble fiber fraction of the FVBP flour before and after the *in vitro* digestion was obtained according to method 991.43 of AOAC (1995) with MES-TRIS buffer. The whole samples were submitted to sequential enzymatic digestion (α-amylase, protease

and amyloglucosidase) and the obtained residue was filtered with hot water (10 mL at 70ºC), ethanol (15 mL - 78%) and acetone (15 mL) and dried in an oven at 105 °C overnight.

The polyphenol content bound to insoluble fiber was determined as described by Xie et al. (2015), with some modifications. The analysis was performed on insoluble residue obtained from FVBP flour before and after the *in vitro* digestion. Initially, 20 mL of ethanol 80% were added to approximately 2 g of sample for removal soluble polyphenol compounds, stirring at room temperature for 1 h and 200 rpm in an orbital shaker (Wiggenhauser, Berlim, Germany). After, the solid residue hydrolysis was carried with 20 mL of 4M NaOH (aq.) and stirring at 37 °C and 250 rpm for 4 h. The hydrolysate was acidified to pH 1.5–2.0 by gradual addition of 6M HCl. After centrifugation at 5000 rpm for 30 min, the supernatant was extracted five times with 30 mL ethyl acetate. The fraction of ethyl acetate was evaporated using a rotary vacuum evaporator (Vacuum Controller V-850, BÜCHI Labortechnik, Flawil, Switzerland) at 30 °C. The resulting residue was then dissolved in 85% ethanol to 10 mL. The extract obtained was stored at −30 °C. The total of polyphenol content present in the extract was determined by the Folin Ciocalteu's method (Singleton & Rossi, 1965) and designated as bound polyphenol content.

2.4.5. Polyphenols profile

The identification and quantification of single polyphenols compounds was carried out in aqueous extract (as described in the item 2.4.1) of each digestion phase aliquots by HPLC-DAD and ultra-violet detection. Separations were performed in a C18 Phenomenex (250 x 4.6 mm x 5 μm particle size (Kromasil) column) and the mobile phases used were acetonitrile 0.2% TFA as solvent A and acetonitrile/water (5:95 v/v) 0.2% TFA as solvent B. The gradient elution used at 1.0 mL/min flow rate was: 100% solvent B at 1 min; 79% solvent B at 30 min; 73% solvent B at 42 min; 42% solvent B at 55 min; and 100% solvent B at 61 min. The injection volume was 20 μL. Identification of the compounds was performed by comparing the retention time and the UV spectra of each compound identified with those of the reference standards. The follow standards were used: p-hydroxybenzoic acid; catequin; epicatechin; vanillic acid; gallic acid; p-coumaric acid; resveratrol; ellagic acid; myricetin; quercetin; kaempferol; caffeic acid; rutin; chlorogenic acid and ferulic acid. The quantification was carried out based on the reference standards, through a calibration curve calculated with different standards stock solution concentrations.

2.5. Antioxidant capacity

The antioxidant capacity was performed with the sample preparation as referred above (item 2.4.1) for analysis of polyphenols compounds in soluble (SF) and pellet (PF) fractions of FVBP flour.

2.5.1. DPPH Radical Scavenging Capacity

The antioxidant capacity of SF and PF aqueous extracts from each digestion step was determined by the radical scavenging ability, using DPPH as a free radical (Brand-Williams et al., 1995). Initially, a solution of DPPH (600 μM) was diluted in ethanol to absorbance of 0.600 ± 0.020, at 515 nm, measured by a spectrophotometer (UV-VIS 1240, Shimadzu, UK). Samples were analyzed by reacting 1.75 mL of DPPH solution (60 μM) with 250 μL of sample for 30 minutes in the dark. After the reaction, the absorbance of three replicates of each sample was determined at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard reference and the result expressed in mg Trolox equivalent / g sample.

2.5.2. ABTS radical scavenging capacity

Determination of antioxidant capacity of SF and PF aqueous extracts through the ABTS assay was performed by the procedure described for Gião et al. (2007). The $ABTS^{\bullet+}$ solution was prepared from mixture of ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) solution at 7 mmol L^{-1} with a solution of potassium persulfate at 2.45 mmol L^{-1}, in a proportion of 1:1 (v/v), and allowed to stir for 16 hours in the dark. After, approximately 1 mL of the $ABTS^{\bullet+}$ solution was diluted in 40 mL of ultra-pure water to obtain absorbance of 0.700 ± 0.020, at 734 nm, measured with a spectrophotometer (UV-VIS 1240, Shimadzu, UK). For analysis of samples, approximately 1 mL of $ABTS^{\bullet+}$ solution was placed to react with 10 μL of the sample for 6 minutes in the dark and absorbance was measured at 734 nm. Ascorbic acid was used as the reference standard and result of three replicates expressed as mg ascorbic acid equivalent / g sample.

2.5.3. Oxygen radical absorbance capacity (ORAC)

For analysis of SF and PF aqueous extracts it was used the procedure described by Ou, Hampsch-Woodill, & Prior (2001), with some modifications. Different concentrations of the SF and PF extracts, with volume of 20 μL in two replicates, were placed in 96-well black microplate to incubation with 120 μl of Fluorescein solution (1166,1 μM) for 10 minutes at 40 ° C in fluorimeter (Fluostar Óptima BMG LABTECH). After, 60 μL of AAPH was added and the microplate placed back into the fluorimeter for approximately 100 minutes at 37°C. As "blanc" a mixture of 20 μL of saline phosphate buffer (PBS 75 mM; pH 7.4), 120 μL of

fluorescein (1166.1 µM) and 60 µL of AAPH (2,2′-Azobis (2-methylpropionamidine dihydrochloride, 48 mM) was used. In addition, 80 µl of PBS and 120 µl of Fluorescein were used as control of the reaction. Also, a calibration curve was performed with 20 µL of different concentrations of Trolox as reference standard, 120 µL fluorescein and 60 uL AAPH. The result was calculated by the mean of two replicates and expressed as µg Trolox equivalent/ mg sample.

2.6. Statistical analysis

The experiments were performed in triplicate and the results expressed as mean ± standard deviation. Differences of mean values were assessed by analysis of variance (ANOVA). Tukey's post hoc test was used to determine the difference of means values at 5% significance level. Pearson's correlation coefficient was applied to determine the correlation coefficients between the samples. All statistical analysis was carried out using IBM SPSS Statistics v21.0 (IBM, Chicago, USA).

3. Results and discussion

Drying of solid byproduct from fruit and vegetable processing and subsequent production of vegetable flour is a viable alternative to extend the storage and the use of this byproduct (Ferreira et al., 2015). In addition, the flour produced from fruit and vegetable byproduct can represent a promising source of bioactive compounds, as dietary fiber and polyphenols (Gonçalves et al., 2018; Gullon, Pintado, Fernández-López, et al., 2015). However, despite a range studies evaluating the composition of agro-industrial byproducts (Abdul Aziz et al., 2012; Can-Cauich et al., 2017; Crizel et al., 2016), there is less information about bioaccessibility and antioxidant properties of bioactive compounds from these byproducts, submitted to *in vitro* gastrointestinal digestion (Gullon, Pintado, Barber, et al., 2015; Lucas-González et al., 2018). Thus, to verify the viability of using the byproduct as a valuable food ingredient, it is essential to assess the impact of gastrointestinal digestion on bioactive compounds and antioxidant properties of the byproduct to determine its nutritional and functional value.

3.1. Changes in free amino acid profile

Amino acids are nutritionally classified as non-essential or essential for humans, according to the endogenously synthesis or not in adequate amount to meet dietary requirement , respectively (Wu, 2016). A total of fifteen free amino acids were identified in FVBP flour (Figure 2) and the total free amino acid content (FAA) was 6.50 ± 0.88 mg/g dry matter, with total essential free amino acids (EFAA) content of 1.05 ± 0.13 mg/g dry matter.

Thus, the ratio of EFAA to total FAA content of FVBP flour was 0.16 ± 0.03, similar to cooked yam flour(0.17) (Zhou & Kang, 2019) but lower than broccoli florets and stalks flour (0.23) (Campas-Baypoli et al., 2009). In general, protein from plant-source food have deficiency in most of essential amino acids content such as histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, compared to animal-source food (Hou et al., 2019; Wu, 2016). However, balanced dietary intake between plant and animal food sources and/or adequate combinations of vegetable-source food, as legumes and cereal intake, can provide most of amino acids and other important nutrients, such as dietary fiber (WHO/FAO/UNU Expert Consultation., 2007; Wu, 2016). Additionally, at a minimum level of nitrogen intake from animal-based proteins, the nitrogen balance improves when this intake is partially replaced by a source of non-essential amino acids, such as plant-based protein, since it avoid that essential amino acids are inefficiently used as source for production of non-essential amino acids (WHO/FAO/UNU Expert Consultation., 2007).

The most abundant FAA in FVBP flour was glutamine (1.56 ± 0.08 mg/g dry matter), followed by asparagine (1.10 ± 0.04 mg/g dry matter), which together represent about 40% of the total FAA in FVBP flour. Glutamine and asparagine are generally most abundant free amino acid in vegetables-source foods, such as pistachio nuts and potato (Hou et al., 2019), and together may represent more than half of non-protein nitrogen fraction (Lee, 2018). It is important to note that glutamine is a structural amino acid that promotes cell growth but, in the absence of glutamine, asparagine is able to promote the cell growth and survival and protein synthesis (Pavlova et al., 2018). In contrast, methionine (0.03 ± 0.004 mg/g dry matter) was the least abundant FAA in FVBP flour and represents less than 1% of the total FAA content. Similarly, low methionine content (< 1% of free amino acid content) was found in florets broccolis flour (Campas-Baypoli et al., 2009) and pulp and peel of quince fruit (Silva et al., 2004). The low content of methionine in FVBP flour, as well as tryptophan (0.06 ± 0.00 mg/g dry matter), nutritionally denominated essential amino acids, was already expected since most of the plant-source foods have, in general, low content of essential amino acids (Hou et al., 2019; Wu, 2016). However, changes in protein digestibility during the digestive process can affect the protein quality and, therefore, it is recommended that protein digestibility, based on individual amino acids digestibility, be evaluated after gastrointestinal digestion (FAO, 2011).

Regarding the impact of *in vitro* gastrointestinal digestion on total FAA content of FVBP flour, no change was observed after the oral and gastric phase ($p>0.05$), compared to

undigested FVBP flour (initial). However, it is recognized that small amounts of FAA are released under oral and gastric conditions, since there are no proteolytic enzymes at oral phase and, in gastric phase, the pepsin, an endoprotease, initiates the proteins hydrolysis into large peptides (Lorieau et al., 2018). In contrast, after the intestine phase, a significant increase ($p<0.05$) of total FAA content of FVBP flour was observed, from 6.49 ± 0.87 mg/g dry matter of undigested sample to 34.24 ± 3.18 mg/g dry matter at intestine phase. The high release of amino acids at intestine phase is expected due the addition of pancreatin, an enzyme extract containing endopeptidases, such as trypsinogen, chymotrypsinogen and elastase, and peptidases such as carboxypeptidase, di- and tripeptidases, which are responsible for release of small peptides and free amino acids at intestinal phase of digestion (Rinaldi et al., 2014). In agreement, was described that the FAA content of coconut meat protein increased by 30.3% with pepsin, and by 86.7% with pancreatin after pepsin (Jin et al., 2015).

Concerning the impact of *in vitro* gastrointestinal digestion on the FAA profile, asparagine and phenylalanine showed a significant increase ($p<0.05$) after gastric phase, by 60% and 127%, respectively. This specific increase can be attributed to the preferential cleavage of pepsin for aromatic residues, such as phenylalanine, with a cleavage probability greater than 40% (Hamuro et al., 2008). After intestine phase, an increase of all identified FAA content in FVBP flour was observed. In addition, the significant increase ($p>0.05$) in the ratio of EFAA to total FAA content of FVBP flour was observed, from about 0.17 to 0.26. Similarly, *in vitro* digestion process promoted an increase in ratio of EFAA to total FAA of cooked yam flour, from 0.17 to 0.26 (Zhou & Kang, 2019). This finding demonstrates an efficient release and potential bioaccessibility of FAA of FVBP flour after *in vitro* digestion and may represent an improvement in the protein digestibility, consequently the protein quality. It is important to note that polyphenols extract from fruits and vegetables may reduce the speed and efficiency of protein digestion (Dufour et al., 2018). In this sense, the results of present study demonstrate that the FVBP flour matrix, even with a high content of polyphenols (Brito et al., 2019), had a positive impact on protein digestion.

Glutamine was the most bioaccessible amino acid in FVBP flour after *in vitro* gastrointestinal digestion, followed by arginine and asparagine, with content of 6.50 mg/g, 4.66 mg/g and 4.20 mg/g dry matter, respectively. Glutamine and arginine have been associated with the regulation of physiological metabolic pathways for maintenance, growth, reproduction and immunity and are considered functional amino acids (Wu, 2009). Additionally, asparagine depletion in the brain may be associated with neurological

impairment (Ruzzo et al., 2013). However, *in vivo* studies should be performed to evaluate the absorption and mechanisms of physiological action of these nutrients from FVBP flour.

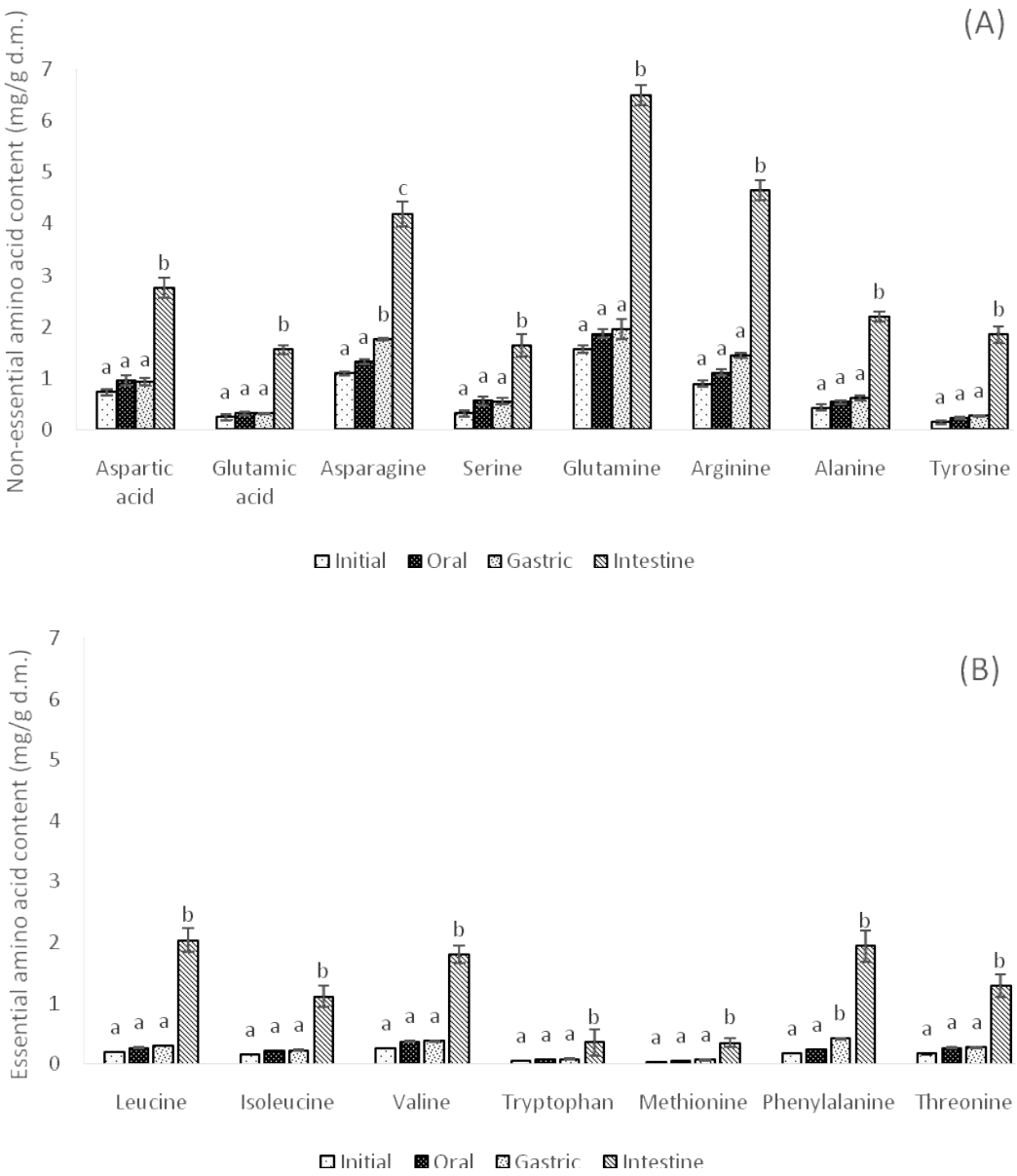

Figure 2. Non-essential (A) and essential (B) free amino acids composition before (Initial) and after *in vitro* gastrointestinal digestion steps (oral, gastric and intestine) of FVBP flour. For the same amino acids, bars followed by the same lowercase letter are not significantly different by Tukey´s test ($p>0.05$). d.m. – dry matter.

3.2. Changes in carotenoid profile

The analysis of β-carotene, lutein and zeaxanthin contents in the FVBP flour and during *in vitro* gastrointestinal digestion is presented in Table 1. Carotenoids are lipophilic compounds found mainly in surface tissues like in peel and outer pericarp of fruits and

vegetables (Kalt, 2005). Thus, by-products from fruits and vegetables may represent a significant source of carotenoids (Abdul Aziz et al., 2012; Silva et al., 2014; Wang et al., 2010). The undigested FVBP flour (Initial) showed highest content of β-carotene (2.11 ± 0.36 mg/100 g) followed by lutein (10.20 ± 0.91 μg/100 g) and zeaxanthin (3.09 ± 0.02 μg/100 g). Similarly, was reported that β-carotene (133.9 μg/g) is the main carotenoid in a mix of fruits and vegetables with their flash and residues, followed by lutein (46.2 μg/g) (Lima et al., 2019). Formulation of fruits and vegetables may have variation in carotenoid content and profile according to the amount, segment, state of ripening and other factors of each fruit and vegetable used (Lima et al., 2019; Niizu & Rodriguez-Amaya, 2005). It is important to note that regular intake of carotenoids may be associated with the prevention of diseases such as cancer, coronary vascular, age-related macular degeneration, cataract formation (Krinsky & Johnson, 2005), maintenance of cognitive health (Johnson, 2012), in addition to protection from sunlight (Stahl & Sies, 2012). However, in addition to the regular intake, the health effects of carotenoids also depends on their bioaccessibility after gastrointestinal digestion (Palmero et al., 2014).

The *in vitro* gastrointestinal digestion showed that β-carotene had the highest stability during digestion, with no variation between all compartments ($p>0.05$). The lutein and zeaxanthin content showed an increase ($p<0.05$) after gastric phase compared to the values of initial FVBP flour and remains similar after intestine phase ($p>0.05$).

Table 1. Total β-carotene, lutein and zeaxanthin before (Initial) and after *in vitro* gastrointestinal digestion phases (oral, gastric and intestine) of FVBP flour.

	β-carotene (mg/100g)	Lutein (μg/100 g)	Zeaxanthin (μg/100 g)
Initial	2.11 ± 0.36^{a}	10.20 ± 0.91^{a}	3.09 ± 0.02^{a}
Oral	1.96 ± 0.07^{a}	9.27 ± 0.08^{a}	3.78 ± 0.22^{a}
Gastric	2.24 ± 0.07^{a}	16.89 ± 0.68^{c}	4.63 ± 0.34^{b}
Intestine	2.40 ± 0.07^{a}	18.54 ± 1.00^{c}	4.85 ± 0.05^{b}

Values are expressed as the mean ± standard deviation. Means values followed by the same lowercase letter within the same column are not significantly different by Tukey´s test ($p>0.05$).

The stability of β-carotene during digestion is recognized in literature, as observed in processed broccoli (Granado-Lorencio et al., 2007) and peach yogurt (Oliveira & Pintado, 2015), as well as higher release and bioaccessibility of xanthophylls (lutein and zeaxanthin) compared to β-carotene (Granado-Lorencio et al., 2007; Kaulmann et al., 2016; Rodríguez-Roque et al., 2013). The higher release of xanthophylls (lutein and zeaxanthin) than carotene in an aqueous environment, such as gastrointestinal tract, is probably related to their structure (Sy et al., 2012; Van Het Hof et al., 2000). Carotenes have a unsaturated hydrocarbon chain

while xantophylls have at least one hydroxyl in their chain, consequently, lower lipophilicity than carotene (Krinsky & Johnson, 2005). In agreement, Schweiggert, Mezger, Schimpf, Steingass, & Carle (2012) reported that the β-carotene bioaccessibility of mango, tomato, papaya and carrot was slightly improved in samples with addition of sunflower oil.

The gastric phase promoted a significant release of lutein and zeaxanthin, by 65% and 50% respectively, from the FVBP flour, compared to the other phase of digestion. Oliveira & Pintado (2015) reported that gastric digestion improved the release of carotenoids from the yogurt matrix and that this fact can be attributed to acid pH and enzymatic activity on the food matrix during this phase of digestion. Carotenoids are known to be unstable at acidic pH, as described by Rodríguez-Roque et al. (2013a) who observed a decrease in the carotene, zeaxanthin and lutein concentration in the gastric phase of *in vitro* gastrointestinal digestion of a blended fruit juice. However, the food matrix can protect some compounds from degradation during gastrointestinal digestion which suggests an influence of food matrix composition and structure on the bioaccessibility of carotenoids (Cilla et al., 2018; Oliveira & Pintado, 2015). Regarding the stability of β-carotene, the interaction with hydrophobic regions of dietary fiber can entrapping them and decrease the bioaccessibility of these compounds (Rodríguez-Roque et al., 2013). In agreement, Palmero et al. (2016) reported that the decrease in bioaccessibility of tomato β-carotene after the rupture of the insoluble fraction at high pressure may be associated with the formed network by polymer-polymer interaction, which entraps the carotenoid-containing fraction.

These findings suggest that carotenoid compounds from FVBP flour, mainly xanthophylls, are released from the food matrix and may be available for absorption after gastrointestinal digestion. The bioaccessibility of zeaxanthin and lutein improved at the end of digestion, while that of β-carotene remained stable compared to the undigested sample, which suggests a protective effect of FVBP flour on the degradation of this compound. It is important to note, however, that *in vivo* studies must be performed to compare and expand the findings of carotenoid bioaccessibility of FVBP flour.

3.3. Total and bound polyphenols content, recovery index and bioaccessibility. of polyphenols

The total polyphenols content (TPC) of FVBP flour before and after *in vitro* gastrointestinal digestion phases is depicted in figure 3 (A). The undigested FVBP flour (initial) showed TPC of 4.83 ± 0.09 mg gallic acid eq./g. A slight decrease ($p<0.05$) of TPC from the initial sample to the oral and gastric phases was observed, without variation between

the oral and gastric phases ($p>0.05$). In contrast, the lowest value of TPC was obtained after the intestine phase, compared to all digestion phases, which represents a decrease about 50% compared to gastric phase ($p<0.05$) and 56% of undigested FVBP flour (initial).

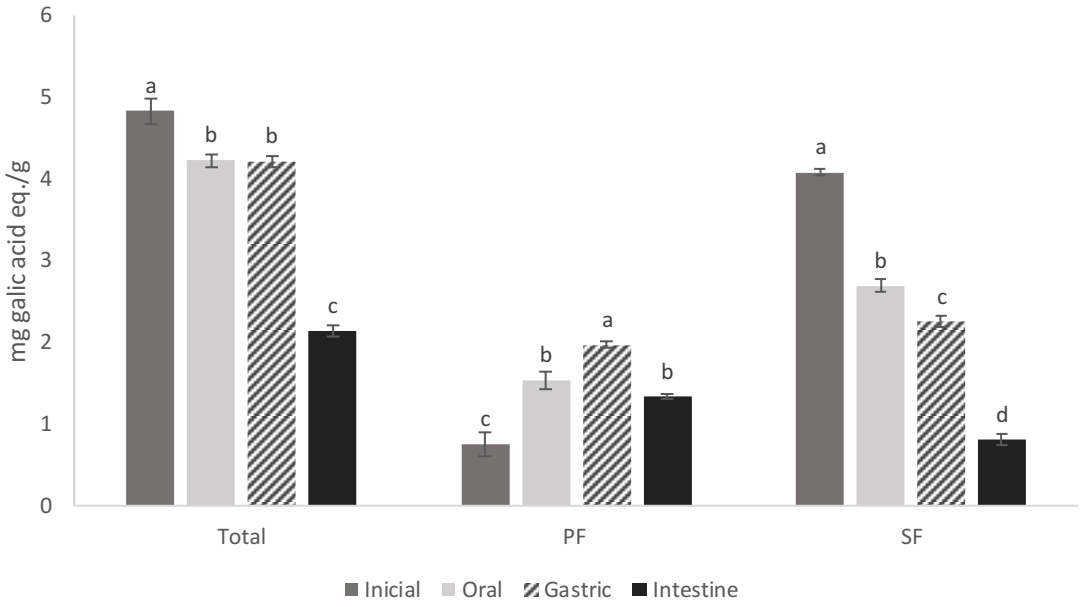

Figure 3. Total polyphenol content (TPC) before (Initial) and after *in vitro* gastrointestinal digestion phases (oral, gastric and intestine) of FVBP flour (Total), FVBP soluble fraction (SF) and FVBP pellet fraction (PF). For the same sample, bars followed by the same lowercase letters are not significantly different ($p> 0.05$) according to Tukey´s test.

The results obtained in the present work reveal a negative effect of *in vitro* digestion on TPC of FVBP flour in all digestion phases, mainly after intestine phase. In this sense, the recovery index of polyphenols shows a slight decrease ($p<0.05$) in oral (87.46%) and gastric (86.95%) phases and the lowest value after the intestine phase (43.89%), compared to all digestion phases, which represents a decrease ($p<0.05$) about 56% of undigested FVBP flour (initial).

The short time of interaction between α-amylase enzyme and the food matrix at oral phase of digestion can lead to a reduced impact on the release of polyphenols compounds from food matrix (Bohn, 2014). In addition, in the oral phase, among other factors, polyphenols compounds are subject to changes due to the interaction with biopolymers, such as fibers, and digestive enzymes (Alminger et al., 2014). In agreement, Lucas-González et al. (2018) reported a decrease of recovery of TPC in the oral phase of persimmon fruit flour *in vitro* digestion, and attributed to the possible interaction of polyphenols compounds and the α-amylase enzyme, and the dietary fiber of persimmon fruit flour.

Regarding to the gastric phase, the remaining ($p>0.05$) of TPC compared to oral phase and decrease ($p>0.05$) compared to initial sample, may be related to the interaction with nutrients such as dietary fiber and peptides released at gastric phase by the action of enzymes such as pepsin and the low pH of the medium (Bohn, 2014; Lucas-González et al., 2018). It is important to note that the presence of dietary fibers during the gastrointestinal digestion is known to impact the gastrointestinal transit time and increase the viscosity of the bulk, which can impair the release of compounds from the complex food matrix (Alminger et al., 2014; Bohn, 2014). A slight decrease of recovery index of TPC at gastric phase of *in vitro* digestion was also described in apple bagasse flour (Gullon, Pintado, Barber, et al., 2015) and persimmon plant fruit peel-fiber digestion (Martínez-Las Heras et al., 2017).

The intestine phase is the last and most extensive phase of digestion, where occur degradation of the food matrix by the action of pancreatic enzymes, as amylases, proteases and lipases, and bile salts (Alminger et al., 2014; Lucas-González et al., 2018). The marked decrease of recovery of TPC from FVBP flour described at this phase ($p<0.05$) agrees with several findings present in scientific literature as in pomegranate peel flour (43%) (Gullon, Pintado, Fernández-López, et al., 2015) and date pits flour (46.02%) (Gullon, Pintado, Barber, et al., 2015). The higher effect of intestine phase of digestion on recovery of TPC may be attributed to the interaction between polyphenols and nutrients such as fibers, proteins and minerals, which may impair the release of polyphenols from food matrix or reduce their solubility (Kroll, Rawel, & Rohn, 2003; Lima et al., 2014; Palafox-Carlos et al., 2011). Additionally, chemical reactions, mainly oxidation and polymerization, may lead to formation of new polyphenol derivatives with lower solubility, which can result in underestimation of total polyphenol content (Gil-Izquierdo et al., 2001; Lucas-González et al., 2018).

The suggested interaction between polyphenols and nutrients released from FVBP flour during *in vitro* gastrointestinal digestion is in accordance with the result obtained for TPC of the soluble (SF) and insoluble (PF) fractions of FVBP flour during the *in vitro* digestion (Figure 3). The SF showed a significant decrease ($p<0.05$) of TPC in all *in vitro* digestion phases, compared to initial test sample (SF), with lowest value of recovery index of polyphenols after intestine phase (0.80 ± 0.11 mg gallic acid eq./g). Thus, at this phase, the recovery index of polyphenols for SF was 19.61%. In contrast, the PF fraction showed an increase ($p<0.05$) in TPC in all digestion phases ($p>0.05$) compared to the initial test sample (PF). The PF shows highest value of TPC on gastric phase (1.96 ± 0.03 mg gallic acid eq./g) and a slight decrease after intestine phase (1.33 ± 0.12 mg gallic acid eq./g). In this sense, the

recovery index of polyphenols of PF shows the highest value after gastric phase (261.33%) followed by oral phase (194.67%) and a slight decrease ($p<0.05$) between the gastric (261.33%) and intestine (177.33%) phases. It is important to note that the decrease of TPC values at intestine phase was higher in the SF than in the PF, which represents a decrease in the solubility of polyphenols compounds after digestion. Thus, the relevant decrease in TPC of SF at the end of *in vitro* digestion is also related to the low bioaccessibility index of polyphenols obtained for FVBP flour in the present work of 37.73%.

The higher recovery of TPC of the insoluble fraction (PF) during *in vitro* digestion and low polyphenol bioaccessibility obtained in the present work can be explained by the interaction between nutrients, mainly dietary fiber, and polyphenols from FVBP flour matrix. This flour has a high fiber content, ca. 48%, with higher insoluble fiber content (39%), and a significant content of available carbohydrates (26%) and proteins (9.5%) (Roberta M.S. Andrade et al., 2016; Ferreira et al., 2015). In agreement, Lucas-González et al. (2018) reported that the low polyphenol bioaccessibility of persimmon fruit flours, about 50%, may be related with multiple interactions between polyphenols and nutrients released from this flour matrix that are rich in insoluble fiber, with a significant content of sugar, protein and minerals. In addition, the interaction between polyphenol and dietary fiber has been described in the literature as by Merve Tomas et al. (2018), who reported a decrease of *in vitro* bioaccessibility of polyphenol of tomato sauce by adding 10% of inulin. In this sense, it is well known that dietary fiber is able to exert a physicochemical entrapment of polyphenol compounds, including those released from the food matrix during digestion, which may reduce the solubility and bioaccessibility of these compounds during *in vitro* digestion (A. E Quirós-Sauceda et al., 2014). However, there was a moderate decrease of recovery of TPC of PF between the gastric and intestine phases that may be related to the presence of bile acids at intestine phase. According to Yang, Jayaprakasha, & Patil (2018), dietary fiber can act on diffusion of polyphenols through physical entrapment but, in the presence of bile acids, the dietary fiber can bind more bile acids, which can promote the release of polyphenols and thus increase their bioaccessibility.

Recently studies have shown that the polyphenols strongly associated with macromolecules (mainly fiber), which are not extractable with aqueous organic solvent (non-extractable polyphenols), represent a significant part of total polyphenol content in matrices such as fruits and vegetables (Matsumura et al., 2016; Pérez-Jiménez & Saura-Calixto, 2015; Rufino et al., 2010), including fruits by-products (Pérez-Jiménez & Saura-Calixto, 2018).

In the present study, bound polyphenol content (BPC) value of insoluble fiber of initial FVBP flour was 2.47 ± 0.08 mg gallic acid eq./g and represents approximately 34% of total extractable and non-extractable polyphenols determined for this sample (7,3 mg gallic acid eq./g). The significant contribution of non-extractable polyphenols was also recently demonstrated by Pérez-Jiménez & Saura-Calixto (2018) in apple and nectarine peels, with 32 and 25% of total polyphenol content, respectively. Similarly, Arruda et al. (2018) reported that insoluble-bound polyphenol compounds were the main polyphenol fraction in araticum fruit pulp (39.96%) and seed (47.38%). Regarding to insoluble fiber fraction, Rufino et al. (2010) reported that significant proportion of polyphenols of acerola and cashew apple fruits is clearly associated with dietary fiber, mainly insoluble fraction.

Concerning the impact of gastrointestinal digestion on BPC of insoluble fiber fraction of FVBP flour, was observed that before digestion the BPC (2.47 ± 0.08 mg gallic acid eq./g) is higher ($p<0.05$) than BPC after digestion (0.28 ± 0.01 mg gallic acid eq./g). Thus only 11% of BPC remaining strongly bound to insoluble fiber of FVBP flour after digestion. Similarly, Saura-Calixto et al. (2007a) also reported that small amounts (10%) of polyphenols of fruits in Spanish diet were inaccessible and remained in food matrix after the whole digestion process.

Dietary fiber may be bound to polyphenol compounds by covalent, non-covalent and hydrophobic bonds, which are individually weak and their formation and disruption occurs in response to small changes, such as pH change in the gastrointestinal tract, leading to release of polyphenols compounds (Palafox-Carlos et al., 2011; A. E Quirós-Sauceda et al., 2014). In addition, the low BPC of insoluble fiber associated with higher recovery of TPC in PF than in SF of FVBP flour obtained in the present work may be related to the multiple interaction of polyphenols and different nutrients of complex food matrix, not only dietary fiber, which may keep the extractable polyphenols incorporated into FVBP flour matrix during *in vitro* digestion. In agreement, using an additional digestion with pancreatic α-amylase, after the gastric and intestinal phases, Velderrain-Roddríguez et al. (2016) identified that about 40% of TPC of mango, papaya and pineapple are incorporated in starch carbohydrates of food matrix, and not dietary fiber.

As seen, the interaction between polyphenols and nutrients from food matrix during *in vitro* digestion may decrease the bioaccessibility of polyphenols in the small intestine but can probably increase the content that reaches the colon. At this phase, polyphenols bound to indigestible fraction can become bioaccessible by the action of the gut microbiota and,

consequently, promote an antioxidant environment in the colon and formation of metabolites derivatives with additional health effects (Jakobek & Matić, 2019; Saura-Calixto, 2011).

3.4. Changes in antioxidant capacity

The total antioxidant capacity of FVBP flour by ABTS assay showed the lowest value after oral phase ($p<0.05$) and the highest value after intestine phase ($p<0.05$). There was an increase ($p<0.05$) about 11% in total antioxidant capacity after the gastric phase and 91% after the intestine phase, compared to the initial sample. In addition, the soluble fraction (SF) of FVBP flour showed a higher antioxidant capacity ($p<0.05$) than the pellet fraction (PF) in all digestion phases by ABTS assay. In agreement, Pellegrini et al. (2017) reported that the highest value of total antioxidant capacity of quinoa seeds was obtained after intestine phase and, after the oral phase, the antioxidant capacity of SF was higher than those of the PF during *in vitro* digestion, by ABTS assay.

Regarding DPPH assay, the total antioxidant capacity of FVBP flour showed the lowest value after oral phase ($p<0.05$). After gastric and intestine phases there was an increase in antioxidant capacity compared to the oral phase ($p<0.05$). However, the DPPH values of gastric and intestine phase showed no difference to the initial sample ($p>0.05$). The SF and PF of FVBP flour showed no difference ($p>0.05$) in antioxidant capacity in the oral phase but PF showed higher antioxidant capacity than SF after gastric phase and lower after intestine phase ($p<0.05$). The decrease of antioxidant capacity after the oral phase by DPPH assay was also described in maqui berry (Lucas-Gonzalez et al., 2016) and in samples of quinoa seeds (Pellegrini et al., 2017). It is important to note that, for all digestion phases, the results of antioxidant capacity in the scientific literature are deeply contradictory but indicate a trend of an increase in antioxidant capacity after the gastric phase and a decrease after intestine phase by DPPH assay (Gullon, Pintado, Fernández-López, et al., 2015; Lucas-González et al., 2018; Wootton-Beard et al., 2011). In addition, similarly to the obtained in the present study, higher antioxidant capacity in the SF than PF after intestine phase was described in quinoa seeds (Pellegrini et al., 2017), apple bagasse flour and date pits flour (Gullon, Pintado, Barber, et al., 2015).

Regarding ORAC assay, similarly to that obtained by ABTS and DPPH assay, the lowest value of total antioxidant capacity of FVBP flour was obtained after oral phase, compared to initial sample. In addition, there was an increase ($p>0.05$) of 99% after the gastric phase and 278% after the intestine phase, compared to initial sample. The antioxidant capacity of the SF was higher than PF in all digestion phases ($p<0.05$). It is important to note

that after intestine phase the SF showed a marked increase of antioxidant capacity of 450% compared to PF and of 367% compared to the SF of initial sample. Similarly, Pavan, Sancho, & Pastore (2014) reported an increase of about 210% in the antioxidant capacity of araticum fruit extract after *in vitro* digestion by ORAC assay. However, different to that obtained in the present study, Gullon, Pintado, Fernández-López, et al. (2015) reported that the ORAC values of PF were higher than the SF values in pomegranate peel flour *in vitro* digestion. Summary, the total antioxidant capacity of FVBP flour (Initial) decrease ($p<0.05$) after the oral phase in any of the evaluated methods (Table 2). After the gastric and intestine phases, an increase ($p<0.05$) was observed by the ABTS and ORAC assay and no difference ($p>0.05$) was observed by DPPH assay, compared to initial FVBP flour sample.

Table 2. Antioxidant capacity of pellet fraction (PF) and soluble fraction (SF) before (Initial) and after *in vitro* gastrointestinal digestion phases (oral, gastric and intestine) of FVBP flour.

Sample		ABTS (mg Trolox eq./g)	DPPH (mg Trolox eq./g)	ORAC (mg Trolox eq./g)
Initial	PF	0.91 ± 0.02^{c}	0.14 ± 0.04^{b}	10.29 ± 1.37^{c}
	SF	3.80 ± 0.03^{e}	0.21 ± 0.003^{c}	22.35 ± 1.47^{a}
	Total	4.71 ± 0.02^{A}	0.35 ± 0.02^{AC}	32.64 ± 1.42^{A}
Oral	PF	1.54 ± 0.02^{d}	0.07 ± 0.01^{a}	7.06 ± 0.55^{c}
	SF	2.64 ± 0.14^{ab}	0.08 ± 0.001^{a}	18.66 ± 1.90^{ab}
	Total	4.18 ± 0.08^{B}	0.15 ± 0.006^{B}	25.72 ± 1.22^{B}
Gastric	PF	2.37 ± 0.24^{a}	0.20 ± 0.005^{c}	26.26 ± 2.32^{a}
	SF	2.87 ± 0.25^{b}	0.12 ± 0.002^{ab}	38.81 ± 1.33^{d}
	Total	5.24 ± 0.24^{C}	0.32 ± 0.003^{A}	65.07 ± 1.83^{C}
Intestine	PF	2.21 ± 0.16^{a}	0.13 ± 0.003^{ab}	18.99 ± 0.87^{ab}
	SF	6.79 ± 0.03^{f}	0.24 ± 0.02^{c}	104.45 ± 2.32^{e}
	Total	9.00 ± 0.09^{D}	0.37 ± 0.011^{C}	123.44 ± 0.59^{D}

PF: Pellet Fraction; SF: Soluble Fraction. Values are expressed as the mean ± standard deviation. Means values followed by the same lowercase letter within the same column are not significantly different by Tukey´s test ($p>0.05$). Means values followed by the same capital letter within the same column are not significantly different by Tukey´s test ($p>0.05$).

The divergent results of the antioxidant capacity of the FVBP flour by different assays, mainly DPPH, may be related to different chemical reactions involved in ABTS, DPPH and ORAC assays. The antioxidant capacity assays are mainly based on the transfer of hydrogen atoms (HAT), as ORAC, or on the transfer of single electron (ET), such as ABTS assay (Huang et al., 2005). The 2,2-diphenyl-1-picryl-hydrazil (DPPH •) radical reacts mainly by ET, however, the HAT reaction can occur in a marginal way (Prior et al., 2005). In addition,

different to DPPH assay, the ORAC and ABTS assays may reflect the lipophilic and hydrophilic antioxidant capacity (Prior et al., 2005). The radical DPPH• is a nitrogen radical, which has no similarity and is less reactive than the peroxyl radicals and may react more slowly or even be inert to some antioxidants (Huang et al., 2005; Wootton-Beard et al., 2011). This fact probably affected the measurement of total antioxidant capacity in the FVBP flour since low inhibition of the DPPH• radical was observed in initial sample, which did not change during the gastric and intestinal phases. It is important to note that color compounds present in plant samples, such as carotenoids, may interfere in decrease of absorbance at low wavelength, such as that performed on DPPH assay (515 nm) and, consequently, less antioxidant capacity is measured (Arnao, 2000).

Regarding ORAC and ABTS assays, higher antioxidant capacity was observed after the gastric and intestine phase of digestion by ORAC, compared to ABTS assay. The ORAC assay is sensitive and can directly reflect the peroxyl radical scavenging activity of vegetables (Ou et al., 2002). In contrast, only compounds with redox potential lower than ABTS can reduce the radical ABTS • + and this reaction can take a long time to reach an endpoint, which can lead to underestimating the ABTS values if the reading is done before the reaction finish (Prior et al., 2005). The higher ORAC values compared to ABTS values after in vitro digestion was also described in pomegranate peel flour (Gullon, Pintado, Fernández-López, et al., 2015) an in araticum, papaya and jackfruit extracts (Pavan et al., 2014).

The correlation between antioxidant capacity and total polyphenol content (TPC) in these phases of digestion is contradictory in the scientific literature (Lucas-Gonzalez et al., 2016; Pellegrini et al., 2017). The results obtained in the present study showed that in the oral phase, DPPH-ABTS-ORAC assays showed a positive moderate correlation ($r > 0.730$). However, the correlation of recovery of TPC of FVBP flour at this phase showed a positive low correlation ($r > 0.439$) with ABTS and negative with DPPH and ORAC assays. These findings suggest a small contribution of TPC to the antioxidant capacity at oral phase measured in the present study. In agreement, Pellegrini et al. (2018) reported a negative correlation between TPC and antioxidant capacity assays after oral phase of chia seeds digestion.

The increase in total antioxidant capacity after the gastric and intestine phases ($p<0.05$) by ABTS and ORAC assays suggests a high release of antioxidant compounds from the food matrix during digestion. However, after gastric phase, TPC showed a strong correlation ($r = 0.999$) with ORAC assay and negative correlation with DPPH and ABTS

assays. The DPPH showed a moderate positive correlation (r = 0.786) with ABTS at this phase. After intestine phase, TPC of FVBP flour showed negative correlation with ABTS-DPPH-ORAC assays. In addition, there was a positive moderate correlation between DPPH and ABTS assays (r = 0.522) and a strong correlation between ABTS and ORAC assays (r = 0.853).

Pellegrini et al. (2017) reported that after gastric phase of *in vitro* digestion of quinoa seeds, the phenolic acids content contributes to a lesser extent to antioxidant capacity by ABTS and DPPH assay, while in the intestinal phase, only ABTS antioxidant capacity was correlated with phenolic acids. In contrast, Lucas-Gonzalez et al. (2016) reported that in gastric and intestine digestion phases there was a high correlation between TPC and the antioxidant capacity of maqui berry measured by DPPH, ABTS, FIC and FRAP assays. Regarding ORAC assay, a strongly correlation was found after intestine digestion phase between TPC and antioxidant capacity in the three carob fractions (Chait et al., 2020) and in pomegranate peel flour (Gullon, Pintado, Fernández-López, et al., 2015).

According to results obtained in the present study, after gastric digestion, there was a high contribution of TPC to the antioxidant capacity of FVBP flour by ORAC assay, and no contribution after intestine phase by all assays. However, there was a slight decrease ($p<0.05$) of recovery index values of TPC (Figure 3) obtained for FVBP flour after gastric digestion phase (86.95%) and a deep decrease after intestine phase (43.89%), compared to initial sample (100%). It is important to note that, according to Oliveira & Pintado (2015), the increase of antioxidant capacity after the intestine phase, in the presence of digestive enzymes, may also be related to non-polyphenols compounds such as amino acids, peptides and soluble sugars released from the food matrix during intestine digestion. Thus, it is suggested that the antioxidant capacity of FVBP flour obtained mainly in the intestine digestion may be also related to other compounds released during *in vitro* gastrointestinal digestion, as vitamins and amino acids.

3.5. Polyphenols profile

Based on the changes observed in the antioxidant capacity of FVBP flour during *in vitro* gastrointestinal digestion, the profile of the main polyphenols compounds in each digestion phase was determined (Table 3). It is important to note that this polyphenol profile is related to the main compounds extracted under similar conditions (temperature, solvent etc.) to those present in the physiological gastrointestinal environment. In this extraction were detected seven phenolic acids (gallic acid, 4-hydroxybenzoic, vanillic acid, syringic acid, p-

coumaric acid, caffeic acid and sinapic acid) and four flavonoids (glycoside and aglycone forms) ((+) - Catechin, (+) - Epicatequin, Quercetin and Kaempferol) (Table 3). Compounds that showed the highest concentration in undigested sample (initial) were (+) – Epicatechin (15.14 ± 0.21 mg/100g), (+) - Catechin (13.08 ± 0.19 mg/100g), followed by gallic acid (12.11 ± 1.19 mg/100g).

Table 3. Polyphenol profile of *in vitro* gastrointestinal digestion steps (mouth, gastric and intestine) of fruit and vegetable residues flour (Initial).

	Concentration (mg/100g dm.)			
	Initial	**Oral**	**Gastric**	**Intestine**
Phenolic acids				
Gallic acid	12.11 ± 1.19^{a}	4.84 ± 0.36^{b}	20.02 ± 1.24^{c}	2.80 ± 0.09^{d}
4-Hydroxybenzoic acid	8.49 ± 0.67^{a}	$8,55 \pm 0.75^{a}$	11.87 ± 0.18^{b}	8.07 ± 1.43^{a}
Vanillic acid	4.21 ± 0.21^{a}	3.34 ± 0.38^{b}	6.95 ± 0.32^{c}	2.18 ± 0.22^{d}
Syringic acid	2.03 ± 0.40^{a}	1.12 ± 0.23^{b}	2.88 ± 0.27^{c}	1.01 ± 0.10^{b}
p-coumaric acid	1.66 ± 0.37^{a}	0.86 ± 0.12^{b}	2.01 ± 0.13^{a}	0.83 ± 0.01^{b}
Caffeic acid	1.23 ± 0.11^{a}	0.65 ± 0.004^{b}	2.61 ± 0.06^{c}	0.43 ± 0.01^{b}
Sinapic acid	0.86 ± 0.04^{a}	2.96 ± 0.02^{b}	3.59 ± 0.55^{b}	2.74 ± 0.34^{b}
Total	**30.59 ± 3.39^{B}**	**22.32 ± 2.05^{BC}**	**49.93 ± 5.03^{A}**	**18.06 ± 1.68^{C}**
Flavonoids				
(+)-Catechin	13.08 ± 0.19^{a}	25.74 ± 0.91^{b}	59.75 ± 1.67^{d}	35.23 ± 0.91^{c}
(+)-Epicatechin	15.14 ± 0.21^{a}	15.78 ± 0.70^{a}	17.71 ± 1.76^{a}	23.47 ± 0.24^{b}
Quercetin	1.84 ± 0.13^{a}	10.25 ± 0.98^{b}	23.63 ± 1.34^{c}	23.97 ± 1.95^{c}
Kaempferol	6.57 ± 0.37^{a}	5.38 ± 0.12^{a}	5.53 ± 0.70^{a}	5.27 ± 0.26^{a}
Total	**36.63 ± 6.09^{C}**	**57.15 ± 6.47^{C}**	**106.62 ± 16.55^{A}**	**87.94 ± 8.36^{B}**

Values are expressed as the mean ± standard deviation. Means followed by the same lowercase or capital letters within the same line are not significantly different ($p>0.05$) according to Tukey´s test. dm. – dry matter.

In regards to oral digestion phase, among the phenolic acids identified, five compounds (gallic acid, vanillic acid, syringic acid, p-coumaric acid and caffeic acid) showed a decrease ($p<0.05$) between 20% and 60% in their concentration, with a marked decrease ($p<0.05$) obtained in gallic acid content (60%), compared to initial sample. In contrast, was found a marked increase ($p<0.05$) in synaptic acid content (244%) and no difference ($p>0.05$) in 4-hydroxybenzoic acid content, compared to initial sample. The decrease of phenolic acids can be explained by the reduced solubility due to interaction with the food matrix and enzyme α-amylase in the oral phase. According to Lucas-Gonzalez et al. (2016), the decrease in all phenolic acids content identified in maqui berry after oral digestion could be explained due to

the possible interaction of these compounds with proteins or fiber in the food matrix, due to the short time exposure time to α-amylase. In addition, the interaction between polyphenols and starch digestive enzymes was also described (Ali Asgar, 2013), which can reduce their solubility. The decrease in most of phenolic acids content in quinoa seeds (Pellegrini et al., 2017) and in gallic acid content in carob products (Goulas & Hadjisolomou, 2019) was also described.

Regarding gastric phase, among phenolic acids, there was an increase ($p<0.05$) in gallic acid, 4-hydroxybenzoic, vanillic acid, syringic acid and caffeic acid content between 40% and 111% and a marked increase in sinapic acid content of 317%, compared to initial sample. It is important to note that, besides this high increase, sinapic acid content showed no difference ($p>0.05$) between the oral and gastric phases while gallic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid and caffeic acid content showed an increase compared to the oral phase ($p<0.05$). In contrast, p-coumaric acid content showed no difference ($p>0.05$) compared to initial sample but an increase ($p<0.05$) when compared to oral phase. This finding suggests that the gastric digestion phase may increase the phenolic acids release from FVBP flour matrix. This increase may be attributed to the hydrolysis induced by acid pH and protease activity during the gastric digestion phase, which may promote the release of polyphenols compounds bounded to other nutrients, as dietary fiber and protein (Rodríguez-Roque et al., 2013; Saura-Calixto et al., 2007). In agreement, the increase in phenolic acids content after gastric digestion phase was described in several works in the scientific literature (Chen et al., 2016; Jara-Palacios et al., 2018; Lucas-González et al., 2018).

It is important to note that the differences observed in the stability between the single polyphenols compounds, in the same digestive conditions, can be related to factors such as their physicochemical properties and the interaction with nutrients (Rodríguez-Roque et al., 2013). Regarding the intestine phase, most of phenolics acids content (gallic acid, vanillic acid, syringic acid, p-coumaric acid and caffeic acid) showed a decrease ($p<0.05$) between 48% and 77% compared to undigested sample (initial). However, the 4-hydroxybenzoic content showed no difference ($p>0.05$) and sinapic acid content showed an increase ($p<0.05$) after the last digestion phase, compared to initial sample. In addition, at intestine phase, the total phenolic acids content decrease ($p>0.05$) compared to the initial phase. The decrease of most phenolic acids content after intestine phase may be associated to the alkaline environment of the intestine, which can lead to the degradation of these compounds (Tagliazucchi et al., 2010). In addition, the interaction with other nutrients from food matrix,

such as fibers, proteins and iron, can reduce the solubility and availability of phenolic compounds (Rodríguez-Roque et al., 2013). Similar to the results obtained in the present study, the negative impact of digestion on the phenolic acid content also described in persimmon fruit flour (Lucas-González et al., 2018) and in the white winemaking byproducts extracts (Jara-Palacios et al., 2018).

The *in vitro* digestion of FVBP flour showed a positive effect on their flavonoids content, with an increase ($p<0.05$) after all digestion phases, compared to initial sample. Regarding single flavonoids, (+) – Catechin content showed an increase ($p<0.05$) in all digestion phases, compared to initial sample, but a decrease ($p>0.05$) between gastric and intestine phase. The increase in (+) – Epicatequin content occur only after the intestine phase ($p<0.05$), compared to other all digestion phases. In addition, quercetin content showed an increase after oral and gastric phase ($p<0.05$), compared to initial sample, but no difference was obtained between gastric and intestine digestion phases ($p>0.05$). Regarding kaempferol content, no difference was obtained ($p>0.05$) between all digestion phases. Different to phenolic acids, flavonoids are stable at pH changes during gastrointestinal digestion (Tagliazucchi et al., 2010). However, the increase of total flavonoids content among gastric and intestine digestion phases can be explained by the effect of digestion enzymes, mainly pancreatin, which also has amylase, lipase and protease activity, that can promote the release of flavonoids bound to the food matrix (Bouayed et al., 2011). In addition, during gastrointestinal digestion, hydrolysis of glycoside forms can occur, producing aglycone forms, which can lead to an increase in flavonoid content (Ortega et al., 2011).

The impact of digestion on the flavonoid content described in the scientific literature is contradictory (Chait et al., 2020; Lucas-González et al., 2018). However, in agreement to the results obtained in the present study, an increase in soluble free flavonoids of carob pulp after digestion was reported by Chait et al. (2020), and in monomers and dimers flavonoids of white winemaking byproducts extracts, reported by Jara-Palacios et al. (2018). The variations found between single compounds and compared to the literature may be related to the chemiodiversity of the bioactive compounds and to the differences in the food matrix composition (Alminger et al., 2014; Rodríguez-Roque et al., 2013).

According to results described in the present study, after the gastric digestion phase the total phenolic acids and flavonoids content showed a strong positive correlation with antioxidant capacity by ORAC ($r = 0.993$ and $r = 1.000$) and ABTS ($r = 1.000$) assays. A strong negative correlation between total phenolic acids content and antioxidant capacity by

ORAC (r = -0.990) and ABTS (r = -0.915) assays was obtained after intestine phase, which corroborates with the overall decrease in single phenolic acids content and recovery of TPC after these phases. Despite this decrease, the antioxidant capacity of FVBP flour increased after the gastric and intestine phases, which may be associated with the increase of single flavonoids content after these digestion phases. The total flavonoids content showed a strong positive correlation with antioxidant capacity by ORAC (r = 0.910) and ABTS (r = 0.999) assays. Similarly, Pellegrini et al. (2018) described that the correlation between TPC of chia seeds and antioxidant capacity assays was negative after oral phase and strongly positive after gastric phase, while the total flavonoids content showed strong positive correlation with antioxidant capacity assays after both of these phases. It is important to note that the Folin-Ciolcateu reagent can react with other non-phenolic compounds such as vitamins, sugars and amino acids, which may underestimate or overestimate the evaluation of TPC by this method (Pellegrini et al., 2018).

Thus, with the results obtained in the present study, it is suggested that under conditions similar to those present in the gastrointestinal tract environment, the bioactive compounds of FVBP flour, mainly phenolic acids, showed lower concentration in the small intestine, compared to the undigested sample. However, a relevant increase in the flavonoid compounds content was obtained after digestion, which represents an increase in availability for the absorption of these compounds.

4. Conclusion

The present work demonstrates the changes in carotenoid, amino acids and polyphenols content and antioxidant capacity of vegetable flour obtained from byproducts of fruit and vegetables processing during *in vitro* gastrointestinal. It is noteworthy that amino acids were efficiently released and high levels of glutamine and arginine were obtained at the end of *in vitro* gastrointestinal digestion of FVBP flour, which can promote beneficial effects as intestinal mucosal trophism.

The total antioxidant capacity of FVBP flour showed an increase after the gastric and intestine digestion phases by ABTS and ORAC assays and suggests a high release of antioxidant compounds from the food matrix during digestion. In addition, the profile of individual polyphenols compounds from FVBP flour demonstrated that, despite the negative impact of *in vitro* digestion on most of phenolic acids content, flavonoid compounds showed greater stability and an increase in concentration after digestion, mainly (+)-Catechin and quercetin.

Thus, it is suggested that the FVBP flour obtained from fruits and vegetables processing, usually discarded by food industry, has potential as a functional ingredient, which can contribute to improving the value of foodstuffs, or as a source of bioactive compounds that have recognized beneficial effect on health.

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4. CAPÍTULO III- Potential prebiotic effect of fruit and vegetable by-products flour using *in vitro* gastrointestinal digestion

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ABSTRACT

Fruit and vegetable byproducts (FVBP) present high content of bioactive compounds and dietary fibers and have demonstrated a positive modulatory effect upon gut microbiota composition. In the present study, the prebiotic potential of a FVBP flour obtained from solid byproducts after fruit and vegetable processing was evaluated after in vitro gastrointestinal digestion. An initial screening with three strains of *Lactobacillus* (*Lactobacillus casei* 01, *Lactobacillus rhamnosus* R11 and *Lactobacillus acidophilus* LA-5®) and one Bifidobacterium strain (*Bifidobacterium animalis* spp. *lactis* BB12®) was carried out and then the prebiotic effect of FVBP flour was performed with fecal samples of five donors. The changes in gut microbiota were evaluated at 0, 12, 24 and 48 h of fermentation by the real-time polymerase chain reaction (qPCR) method with 16S rRNA-based specific primers. The pH and short chain fatty acids (SCFA) production at each fermentation time were assessed. The fructooligosaccharides (FOS) were used as positive control. The impact of FVBP flour upon cell viability was also evaluated. FVBP flour showed higher prebiotic effect than FOS on growth enhancement of Lactobacillus after 48 h of fermentation and similar bifidogenic effect as FOS on Bifidobacterium growth at 12, 24 and 48 h of fermentation. SCFA production was observed when FVBP flour was used as carbon source, including butyrate, which supports the prebiotic potential of this flour. Additionally, it was observed that after in vitro gastrointestinal digestion, the FVBP flour at 3% promoted cell metabolism of Caco-2 cell line up to 67%. Thus, the present study demonstrates the viability of using a fruit and vegetable byproducts flour as a potential sustainable prebiotic source.

Keywords: fruit and vegetable; residues; functional flour; dietary fiber; in vitro digestion; human fecal microbiota; prebiotic property.

1. Introduction

Fruit and vegetable industrial processing has increased to meet growing consumer demand for healthy products (Sagar et al., 2018). This has generated a large amount of plant byproduct (i.e., bagasse, peels, seeds, leaves, etc.), which composes up to 60% of the raw material processed and is commonly discarded (Amaya-Cruz et al., 2015). Disposal of this material not only poses an environmental problem but also represents a loss to human health of important nutrients. An important global challenge currently is the search for environmentally sustainable food systems. Fortunately, an increasing number of strategies are aimed at efficient processing and obtaining quality value-added products from the starting material (FAO, 2018; Pérez-Jiménez & Viuda-Martos, 2015).

Byproducts generated during fruit and vegetable juice processing, for example, have significant dietary fiber (Andrade et al., 2016; Gouw et al., 2017) and phytochemical content (Gonçalves et al., 2018; Amaya-Cruz et al., 2015). The fruit and vegetable byproduct flour (FVBP flour), obtained from solid residues generated in fruit and vegetable processing, shown to have a high insoluble fiber fraction (39%) and an available carbohydrate (mono- and disaccharides plus starch) content of 26%, as well as a significant soluble fiber fraction (9.6%) and protein content (9.5%) (Andrade et al., 2016; Ferreira et al., 2015). This flour also presented a rich polyphenols profile, containing 28 phenolic acids, 32 flavonoids and 28 other polyphenols compounds (Gonçalves et al., 2018). This byproduct is therefore a rich source of bioactive compounds and a functional ingredient. A study of the functional capacity of FVBP flour showed an improvement in constipation symptoms in 87.5% of female volunteers after 10 days of daily consumption of 10 g of FVBP flour, which is possibly associated with high insoluble fiber content of this food matrix. In this sense, studies to explore the impact of a complex food matrix on the modulation of gut microbiota may extend the use of byproducts as functional food ingredients and improve the nutritional characteristics of foodstuff.

More recently, interest in plant fiber-rich byproducts has increased due to their demonstrated positive modulatory effect upon gut microbiota composition, which gives them prebiotic potential (Albuquerque et al., 2019; Sáyago-Ayerdi et al., 2019). A prebiotic can be defined as a substrate that is selectively utilized by host microorganisms to confer a health benefit (Gibson et al., 2017). Dietary fibers and other non-digestible carbohydrates that reach the intestine are susceptible to bacterial fermentation and thus may influence the composition and metabolic activities of gut microbiota (Holscher, 2017). Short-chain fatty acids (SCFA) such as acetate, butyrate, and propionate are fermentation end products that

may have beneficial local and systemic effects upon host health when they are produced in the intestine (Rastall & Gibson, 2015).

Potential prebiotic effects have been associated with a rich composition of oligosaccharides such as fructooligosaccharides (Diaz-Vela et al., 2013) and pectin oligosaccharides (Gómez et al., 2019), which are considered potential prebiotics (Cantu-Jungles et al., 2017; Karboune & Khodaei, 2016). Additionally, researchers have recognized the potential prebiotic effect of polyphenols from fruits and vegetables on improving the growth of healthy target bacteria (Tuohy et al., 2012). To establish that a product has a potential prebiotic effect, it is necessary to evaluate its influence upon gut microbiota composition, namely through the stimulation of butyrate-producing bacteria or the production of metabolites that stimulate butyrate production by other microbiota, which is called the cross-feeding effect (Gibson et al., 2017; Sáyago-Ayerdi et al., 2019).

Studies on humans have shown that the effects of non-digestible carbohydrates upon gut microbiota composition can vary greatly between individuals (Gibson et al., 1995). This can be explained by the large variability in individual gut microbiota composition, which may be influenced, for example, by age (Claesson et al., 2011), type of diet (Singh et al., 2017), and geographical location (De Filippo et al., 2010). *In vivo* tests are the best models for investigating the effects of food on gut microbiota; however, they are time-consuming, costly, and subject to many potential ethical restrictions (Macfarlane & Macfarlane, 2007). In contrast, *in vitro* models are useful for simulating digestion and fermentation of nutrients in the gastrointestinal tract and have the advantages of being faster and less expensive while allowing for a large number of substrates and/or fecal samples to be tested at once (Macfarlane & Macfarlane, 2007). To evaluate changes in gut microbiota, we used the real-time polymerase chain reaction (qPCR) method with 16S rRNA-based specific primers (Healey et al., 2017; Reichardt et al., 2018). This has the advantage of being fast and allowing for the detection of specific species, genera, or groups within a complex bacterial population such as human stool samples (Moon et al., 2016).

Growing evidence from recent studies suggests that bioactive compounds and nutrients derived from byproducts influence gut microbiota. However, most of these studies have used isolated fractions or extracts of dietary fiber or oligo- and polysaccharide to assess prebiotic potential (Cantu-Jungles et al., 2017; Garcia-Amezquita et al., 2018; Gómez et al., 2019). Our study evaluates the *in vitro* prebiotic potential of -byproduct flour generated from fruit and vegetable processing, using qPCR to assess the growth of probiotic strains in a

human fecal model after *in vitro* gastrointestinal digestion has taken place. We also monitored the impact of digested flour on gut cell viability.

2. Material and methods

2.1. Sample

The fruit and vegetable byproducts flour (FVBP flour) was obtained from solid byproducts of fruit and vegetable processing for an isotonic drink development, as described by Ferreira et al. (2015). The solid byproducts were dried in an oven at 65 ° C for 6 h and then ground for 5 min for obtained the FVBP flour with an average particle size of 350 μm (Ferreira et al., 2015; Andrade et al., 2016). Its chemical composition is shown in table 1. The moisture, ash, crude fat, crude fiber and protein (N × 6.25) contents were determined by Ferreira et al. (2015) according to standard methods (AOAC, 1984). The content of available carbohydrates was determined by difference from the protein, fat, moisture, ash, and crude fiber contents (Ferreira et al., 2015). The total, soluble and insoluble dietary fiber fractions were determined by Andrade et al. (2014) according to the AOAC Method 991.43 based on the enzymatic gravimetric procedure (AOAC, 2000). The cellulose, holocellulose (cellulose + hemicelluloses) and lignin content were determined by Brito et al. (2019) according to Sun et al. (2004), and to the TAPPI T19 om-54 (TAPPI, 2002a) and TAPPI T 222 om-02 standards (TAPPI, 2002b), respectively (Brito et al., 2019). The total and resistant starch contents were determined by Brito et al. (2019) according to the standard methods (AACC, 2010; AOAC, 2002) using K-Rstar kit reagents (Megazyme International, Ireland).

The FVBP flour was composed by the byproducts from processing of following species: Selecta orange (Citrus sinensis), passion fruit (Passiflora edulis), watermelon (Citrullus lanatus), lettuce (Lactuca sativa), courgette (Cucurbita pepo), carrot (Daucus carota), spinach (Spinacea oleracea), mint (Mentha sp), taro (Colocasia esculenta), cucumber (Cu- cumis sativus), and rocket (Eruca sativa).

Table 1. Chemical composition of Fruit and vegetable by-product (FVBP) flour.

Composition (% d.b.)	FVBP flour	Reference
Moisture*	5.88 ± 0.49	(Ferreira et al., 2015)
Ash*	4.93 ± 0.41	
Protein*	9.52 ± 1.68	
Fat*	5.09 ± 0.50	
Available Carbohydrates (mono- and disaccharides plus starch)	26.00	
Total dietary fiber	48.42 ± 1.43	(Roberta M. S. Andrade et al., 2016)
Soluble dietary fiber	9.56 ± 0.88	
Insoluble dietary fiber	38.82 ± 0.55	
Insoluble lignin**	14.9 ± 1.6	(Brito et al., 2019)
Cellulose**	19.1 ± 1.3	
Hemicellulose**	6.5 ± 1.0	
Soluble lignin**	5.0 ± 1.4	
Total starch**	14.3 ± 0.4	
Resistant starch**	0.7 ± 0.0	

Values are expressed as means ± standard deviations *Percentage calculated from the mean ± standard deviation expressed as $g.Kg^{-1}$ d.b from Ferreira et al. (2015). **Percentage obtained from the mean ± standard deviation expressed as g/100 g of FVBP flour with granulometric size of 212-300 μm from Brito et al. (2019). d.b – dry basis

2.2. *In vitro* gastrointestinal digestion

FVBP flour was submitted to an *in vitro* gastrointestinal digestion according to Minekus et al. (2014) carried out in four different phases, the oral, gastric and intestinal digestion simulation followed by dialysis (to mimic the intestinal absorption), performed in duplicate. Initially, 22.5 g of the sample were suspended in 150 mL of tap water and pH value was adjusted to 6.0 ± 0.02 using HCl (1 M). The oral phase was simulated by the addition of 1.2 mL of artificial saliva (117.5 U/mL α-amylase (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) and the suspension was incubated for 2 min at 37 °C in a shaking water bath (200 rpm) (Julabo GmbH, Seelbach, Germany). For the gastric phase, the pH was adjusted to 2.0 with HCl (1 M) and pepsin (800–1000 U/mg protein) (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) was added at a ratio of 0.05 mL/mL of solution. After, the digesta solution was incubated for 120 min, 130 rpm and 37 °C. Afterwards, to simulate the intestine phase, the pH value was adjusted to 6.0 using $NaHCO_3$ (1 M) and a mixture of 4 x USP pancreatin (2 g/L, based on trypsin activity) (Sigma-Aldrich Chemistry, St. Louis, Missouri,

USA) and bile salts (12 g/L) (Oxoid ™, Hampshire, UK) was added at a ratio of 0.25 mL/mL of digesta solution and incubated (120 min, 45 rpm, 37 °C). Subsequently, dialysis was simulated using a semipermeable dialysis membrane (Biotech Cellulose Ester Dialysis Membranes, Spectrum Laboratories, Inc., USA) with molecular weight cut-off of 500 Da. The membranes containing the sample solution were immersed in distilled water, under constant stirring, for 48 h at room temperature (25 °C). The pH was measured in each phase using a pH meter (Crison Instruments, Barcelona, Spain) with a Hach 52-07 pH electrode (Loveland, USA).

The content of dialysis membranes was used for screening of potential prebiotic effect and for gut microbiota fermentation analysis. For this, the content of dialysis membrane was freeze dried (Christ freeze dryer Alpha 1-4, Osterode Am Harz, Germany) and the resulting powder stored in desiccator with silica at 25 °C until use in the gut microbiota fermentation. For screening of potential prebiotic effect, the content of dialysis membrane was centrifuged at 4 °C and 4,200 x *g* for 15 min to separate the supernatant and solid fractions. The supernatant was denominated soluble fraction (SF) and the solid fraction obtained was called pellet fraction (PF). Both fractions were freeze dried and stored in a desiccator for use in screening of potential prebiotic effect. All assays were performed in duplicate.

2.3. Screening of prebiotic effect

The potential prebiotic effect of the freeze dried soluble supernatant fraction (SF) and solid pellet fraction (PF) of FVBP flour dialysis membrane content was determined by screening their impact upon the growth of three potential probiotic *Lactobacillus* strains (*Lactobacillus casei* 01 (Chr. Hansen, Hørsholm, Denmark); *Lactobacillus rhamnosus* R11 (Lallemand, Montreal, Canada); *Lactobacillus acidophilus* La-5® (Chr. Hansen, Hørsholm, Denmark) and one *Bifidobacterium* strain (*Bifidobacterium animalis* spp. *lactis* BB12® (Chr. Hansen, Hørsholm, Denmark). The strains were stored at -80 ºC in De Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) with 30% (v/v) glycerol. All the strains were used as monoculture and, before the assays, were grown in MRS broth at 37 °C for 24 h under aerobic conditions for *Lactobacillus* strains and under anaerobic conditions for *B. animalis* BB12, using an anaerobic workstation (Whitley DG250 Don Whitley Scientific, Bingley, UK). The SF and PF and fructooligosaccharides (FOS, positive control) (Sigma-Aldrich Chemistry, St. Louis, USA). were added to sterilized basal media for fermentation at a concentration of 3% (w/v). The basal media was used as negative control (NC) and was comprised of 5.0 g/L of Tryptone Soy Broth without dextrose (Biokar Diagnostics, Beauvais,

France), 5.0 g/L of Bacto™ Peptone (BD Biosciences, New Jersey, USA), 0.5 g/ L of Difco™ Yeast Nitrogen Base (BD Biosciences, New Jersey, USA), 0.5 g/L of cysteine-HCl (Merck, Darmstadt, Germany), 1.0% (v/v) saline solution A [100.0 g/L NH_4Cl (Merck, Darmstadt, Germany), 10.0 g/L $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany), 10.0 g/L $CaCl_2 \cdot 2H_2O$ (Carlo Erba, Chaussée du Vexin, France)], 1.0% (v/v) of trace mineral solution (ATCC, Virginia, USA), 0.2% (v/v) saline solution B [200.0 g/ L K_2HPO_4. $3H_2O$ (Merck, Darmstadt, Germany)] and 0.2% (v/v) of a 0.5 g/L resazurin solution (Sigma-Aldrich Chemistry, St. Louis, USA).

The basal media for fermentation with added samples (SF, PF and FOS) and without any added compounds (NC) was inoculated with 1×10^6 CFU/mL of each strain and incubated at 37 °C for 48 h. For *B. animalis* BB12, the culture medium was anaerobically incubated (85% N_2, 10% CO_2 and 5% H_2) in an anaerobic workstation.

In order to determine the viable bacterial counts, 1 mL aliquots of culture media were collected before and after fermentation (0 h and 48 h) and serially diluted. After, were plated in MRS agar (Biokar Diagnostics, Beauvais, France) (with addition of cysteine hydrochloride (Merck, Darmstadt, Germany) for *B. animalis* BB12) and incubated at 37 °C for 48 h. The increase in viable bacterial counts after 48 h of fermentation was calculated according to the following equation:

$$Increase\ of\ bacterial\ number = Log\ (N/No) \qquad \text{Eq. (1)}$$

where, No is the bacterial number at 0 h (CFU/mL), and N is the bacterial number after fermentation for 48 h (CFU/mL).

2.4. *In vitro* fecal fermentations

2.4.1. Fecal sample collection

Fecal sample donors were selected through the following inclusion criteria: age between 18 to 65 years old; no restrictive diet (e.g. vegetarianism); no food intolerances or severe food allergies and no used of prebiotic supplements, probiotics or antibiotics in the last 6 months. Additionally, all donors must have signed an informed consent form.

The five donors (A-E) selected, three men and two women with ages between 23 and 63 years, received specific instructions for sample collection in addition to a suitable hygienic collection/storage kit. Fresh fecal samples were analyzed no later than 2 h after storage. Fecal inoculum was prepared by diluting the individual feces to 100 g/L in Reduced Physiological

Salt (RPS) solution consisting of 0.5 g/L cysteine-HCl and 8.5 g/L NaCl (LabChem, Zelienople, USA) in an anaerobic workstation.

2.4.2. Fermentation medium preparation

The basal medium for fermentation consists of the same formulation previously described (2.3). Initially, 50 mL of basal medium for fermentation was added to glass bottles with pH value adjusted to 6.8, and the solution bubbled with N_2 until a yellowish color was obtained. FOS were used as positive control, and pre-digested and freeze-dried FVBP flour were added to bottles with basal medium, to a final concentration of 20 g/L. Then, the glass bottles were covered and sterilized. Afterwards, and prior to addition of fecal inoculums, the atmosphere of each bottles was refluxed with a gaseous mixture (10 % CO_2, 5 % H_2 and 85% N_2) sterilized using a 0.22 μm filter (Millipore, Burlington, USA).

2.4.3. Fecal fermentations

Approximately 2% (v/v) of each fecal inoculum (section 2.4.1) was added to the previously prepared bottles containing basal medium and samples were incubated for 48 h at 37 °C, inside an anaerobic workstation. The positive and negative controls were respectively designated as FOS and C (fecal inoculum only), while the pre-digested fruit and vegetable by-product flour was denominated FVBP flour. Aliquots of each sample were collected on 0, 12, 24 and 48 h fermentation and centrifuged for 6 min and 4,000 x *g*. The resulting supernatants were used to evaluate the production of organic acids, while the pellet fraction was used to extract genomic DNA (gDNA). The study was conducted to determine members of the autochthonous microbiota of fecal samples. All assays were performed in triplicate. At each sampling point, the pH values were measured in duplicate and subsequently, the samples were stored at -30 °C for further analysis.

2.4.4. DNA extraction

The extraction of gDNA was performed by using NZY Tissue gDNA isolation kit (NZYTech, Lisbon, Portugal), according to the manufacturer's instructions with some modifications. Initially, the resulting pellet fraction from fecal fermentation was washed 3 times with Tris-EDTA (TE) buffer (pH 8.0), vortexed for 1 min and centrifuged (4.000 x *g*, 10 min). Subsequently, 180 μL of lysozyme solution (10 mg/mL of lysozyme in a 30 mM NaCl and 10 mM EDTA solution) were added to the washed pellet and the solution incubated for 1 h at 37 °C. Then, 350 μL of NT1 buffer was added to the samples, vortexed for 1 min and incubated at 95 °C. After 10 min, samples were centrifuged at 11,000 x *g* for 10 min at 4 ° C. To the resulting supernatant, 25 μL of proteinase K was added and incubated at 70 °C for

10 min. All remaining phases were performed according to the manufacturer's instructions. The concentration of gDNA was assessed using a microplate photometer (μDrop plate, Multiskan FC microplate, Thermo Fisher Scientifc, Waltham, USA).

2.4.5. Real-time polymerase chain reaction (qPCR)

The qPCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA). Each qPCR reaction contained 5 μL of 2x iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, USA), 2 μL of ultrapure water, 1 μL of sample gDNA (equilibrated to 20 ng/μL) and 1 μL of forward and reverse primers (100 nM) targeting the 16S rRNA gene. The primers (STABvida, Lisbon, Portugal) used are listed in table 2, along with the specific annealing temperature.Conditions were as follows: hot start at 95 °C for 10 min followed by 45 cycles of denaturation (95 °C for 10 s), annealing and extension (72 °C for 15 s). As a control of PCR quality, a melting curve analysis was performed for each PCR, using temperatures ranging from 60 to 97 °C, with an increase of 0.1 °C per 0.01 min. All assays were performed in quadruplicate and standard curves were generated using serial dilutions of bacterial gDNA standards of *Lactobacillus gasseri* (ATCC 33323), *Bacteroides vulgatus* (ATCC 8482), *Clostridium leptum* and *Bifidobacterium longum* subsp. *Infantis* (ATCC 15697) (DSMZ, Braunschweig, Germany).

Table 2. Sequences of primers targeting bacterial groups, PCR product size (bp) and annealing temperature (°C). Adapted from Marques et al. (2016)

Target group	Primer sequence (5'-3')	Genomic DNA Standard	PCR Product size (bp)/ Annealing temperatures (°C)	References
Firmicutes	FATG TGG TTT AAT TCG AAG CA R AGC TGA CGA CAA CCA TGC AC	*Lactobacillus gasseri (*ATCC 33323)	126 bp/ 45°C	(Queipo-Ortuño et al., 2013)
Bacteroidetes	F CAT GTG GTT TAA TTC GAT GAT R AGC TGA CGA CAA CCA TGC AG	*Bacteroides vulgatus* (ATCC8482)	126 bp/ 45°C	(Queipo-Ortuño et al., 2013)
Bacteroides	F ATA GCC TTT CGA AAG RAA GAT R CCA GTA TCA ACT GCA ATT TTA	*Bacteroides vulgatus* (ATCC 8482)	495 bp/ 45°C	(Matsuki et al., 2004)
Clostridium leptum subgroup	F GCA CAA GCA GTG GAG T R CTT CCT CCG TTT TGT CAA	*Clostridium leptum*	239 bp/ 45°C	(Matsuki et al., 2004)
Lactobacillus	F GAG GCA GCA GTA GGG AAT CTT C R GGC CAG TTA CTA CCT CTA TCC TTC TTC	*Lactobacillus gasseri* (ATCC 33323)	126 bp/ 45°C	(Delroissea et al., 2008)
Bifidobacterium	F CGC GTC YGG TGT GAA AG R CCC CAC ATC CAG CAT CCA	*Bifidobacterium longum subsp. Infantis* (ATCC 15697)	244 bp/50°C	(Delroissea et al., 2008)

bp – base pairs. F - forward primer; R - reverse primer.

2.4.6. Short chain fatty acids (SCFAs) and lactate production

The resulting supernatants of fecal fermentation were filtered through a 0.22 μm syringe filter for analysis of organic acid production using a HPLC system containing a Knauer K-1001 pump (Berlin, Germany), with a UV-vis detector (220 nm) and a refractive index detector (Knauer, Berlin, Germany). Aliquots of 40 μL of filtered sample were injected directly in an Aminex HPX-87H ion exchange (300 x 7.8 mm) (Bio-Rad, Hercules, USA) column. The column temperature was 65 °C and 13 mM sulfuric acid (Merck, Darmstadt, Germany) was used as mobile phase, at a flow rate of 0.6 mL/min. All assays were carried out in duplicate. Chromatographic peaks were identified in comparison to the retention time of reference standards (acetate, propionate, butyrate and lactate) and quantified by standard's curve regression formula.

2.5. Impact upon cell viability

2.5.1. Cell lines and general growth conditions

Caucasian colon adenocarcinoma cells (Caco-2) were obtained from the European Collection of Authenticated Cells Cultures (ECACC 8601020) through Sigma-Aldrich (St. Louis, USA; ECACC) (reference 09042001). Cells were grown using high glucose Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS; Biowest, France), 1% (v/v) Pen-Strep (Lonza, Basel, Switzerland) and 1% (v/v) of non-essential amino acids 100x (NEAA; Lonza, Basel, Switzerland). All cells were incubated at 37 °C in a humidified atmosphere incubator with 5% CO_2.

2.5.2. Cell viability determination

The impact of FVBP flour before and after *in vitro* gastrointestinal digestion upon Caco-2 cells was evaluated through the sodium salt of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT). Briefly, a 10 mM of phenazine methosulfate (PMS) solution (Sigma-Aldrich, St. Louis, USA) was prepared in phosphate buffered saline (PBS, 0.01 M, pH 7.4) and a 1 mg/mL XTT solution was prepared using DMEM high glucose, previously warmed to 37 °C. Both solutions were sterilized using a 0.22 μm sterile membrane filter (Millipore, Billerica, USA) and mixed, immediately before being used (2.5 μL of PMS per mL of XTT solution).

Caco-2 cells were seeded onto a 96-well microplate (Nucleon Delta Surface, Thermo Scientific, Roskilde, Denmark) at a concentration of 1 x 10^4 cells/well and allowed to adhere. After 24 h incubation the media was removed and replaced with culture media containing the

lyophilized flour sample before and after the digestion at a concentration of 30 and 60 mg/mL (v/v). After 24 h of incubation in the previously described conditions, 25 µL of XTT-PMS solution was added to each well. The plate was incubated in the dark for 2 h and the OD at 485 nm was then measured using a microplate reader (FLUOstar, OPTIMA, BMG Labtech, Ortenberg, Germany). All assays were performed in quintuplicate.

2.6. Statistical analysis

The Shapiro-Wilk's test was used to evaluate the normality of data's distribution. The difference of mean value between each sample (control negative, control positive and FVBP flour) in each bacterial population at each time was assessed by Analysis of Variance (ANOVA). Tukey's post hoc test was used to determine the difference of means values at 5% significance level. All statistical analysis was carried out using IBM SPSS Statistics v21.0 (IBM, Chicago, USA).

3. Results and discussion

The benefits of a prebiotic substrate depend upon its initial use for fermentation by specific organisms in the gastrointestinal tract and its subsequent selective influence on the growth of potentially beneficial microorganisms like *Bifidobacterium* and *Lactobacillus* strains (Huebner et al., 2007). Therefore, the prebiotic potential of a specific substrate can be evaluated by using the growth of these microorganisms as one of parameters. Figure 1 presents data on the prebiotic potential of PF, SF, and controls, assessed after *in vitro* gastrointestinal digestion.

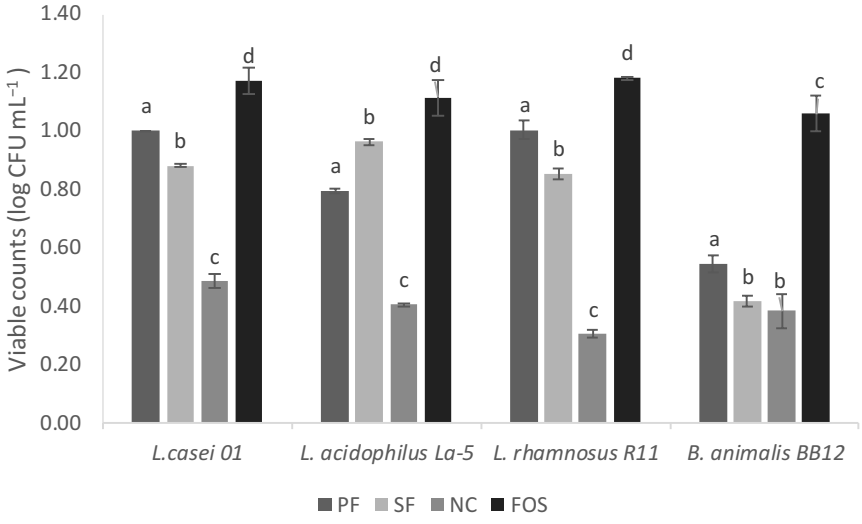

Figure 1. Total viable counts (log CFU mL^{-1}) of *L. casei* 01, *L. acidophilus* La-5, *L. rhamnosus* R11 and *B. animalis* BB12 fermentation for 48h with pellet fraction (PF) (3%), soluble fraction (SF) (3%), FOS (3%) (Positive control) and without an added carbon source (Negative control - NC). Bars followed by the same lowercase letter (within bacterial strain) are not significantly different by Tukey´s test ($p > 0.05$).

It was observed that, compared to the negative control, an increase of viable cell count occurs for PF, SF, and FOS ($p < 0.05$), with the exception of SF in *B. animalis* BB12 ($p > 0.05$). In addition, PF showed a greater ($p < 0.05$) impact than SF on *L. casei* 01, *L. rhamnosus* R11 and *B. animalis* BB12 growth. In summary, the number of viable cells increases in the order of carbon sources FOS>PF >SF>NC. Regardless of the order, this demonstrates that each of the samples at 3% concentration may allow for the growth of potentially beneficial microorganisms.

The differences between SF and PF for *Lactobacillus* strains' growth can be attributed to differences in carbohydrate metabolism resulting from the genomic and metabolic diversity of *Lactobacillus* (Martino et al., 2016; Watson et al., 2013). In contrast, it is well described that *Bifidobacterium* strains may show higher growth rates in medium with specific short-chain oligosaccharides (like FOS) than in the presence of monosaccharides and long chain oligosaccharides (Moniz et al., 2016; Rossi et al., 2005). Moreover, *Bifidobacterium* can use various non-digestible carbohydrates, such as oligo- and polysaccharide fractions of plant cell walls, making these compounds emerging potential prebiotics (Cantu-Jungles et al., 2017; Gómez et al., 2019). Thus, among FVBP flour fractions, the greater ($p < 0.05$) result observed for PF on *Bifidobacterium* growth may be explained by the FVBP flour composition, which has a high insoluble fiber content (39%), primarily cellulose (19.1%) and insoluble lignin (14.9%), as well as a significant soluble fiber content (9.6%) (Andrade et al., 2016; Brito et al., 2019; Ferreira et al., 2015). In addition, the observed difficulty in solubilizing and mixing FVBP flour in digested solution is probably associated with flour's high dietary fiber content. The increased viscosity of the solution, mainly due to soluble and insoluble polysaccharides, can slow the release and solubilization of compounds during digestion (Alminger et al., 2014).

Higher production of butyrate compared with a control has also been regarded as an important indicator of substrate's potential prebiotic effect when it is shown that health-related microorganisms selectively used this substrate (Gibson et al., 2017). Butyrate is the primary energy source for colonocytes and plays a key role in maintaining both the integrity of the intestinal epithelium and the stability of the gut microbiota (X. Wu et al., 2018). The butyrate content and pH change during 48h of fermentation for PF, SF and control's samples are shown in Figure 2. For all bacterial strains, there was an increase ($p<0.05$) in butyrate production when PF and SF were used as carbon sources, compared to the negative control. In contrast, FOS promoted a significant increase ($p<0.05$) in butyrate production only in *L. casei*

01 and *B. animalis* BB12. It is important to note that production of butyrate was similar ($p>0.05$) when FOS and PF were used as carbon sources by BB12.

Similarly, Costa et al. (2019) reported that the production of butyric acid by fermentation with *L.* casei 01 was higher when the carbon source used was grape seed extract compared to FOS. In this same study, the authors reported that production of butyric acid by fermentation with BB12 was not affected by grape seed extract and FOS (Costa et al., 2019). The physicochemical properties of the substrate, such as chain length and degree of polymerization, may affect its use by various microorganisms and consequently impact the production of fermentation end products (Holscher, 2017; Louis et al., 2007). In our study, the composition of FVBP flour, rich in both insoluble fibers like lignin and cellulose, and soluble fibers such as soluble lignin (Andrade et al., 2016; Brito et al., 2019) may explain the stimulation of butyrate production by both *Lactobacillus* and *Bifidobacterium* strains.

SCFAs produced by bacterial fermentation can reduce pH in the colon; this change can then be used as an indicator of bacterial fermentation (Holscher, 2017). We observed a significant reduction ($p<0.05$) in pH after 48 h of fermentation with all bacterial strains assessed using PF and FOS as carbon sources, compared to the negative control (Figure 2). For SF, pH reduction from fermentation by *L. rhamnosus* R11 and *B. animalis* BB12 was similar ($p>0.05$) to the negative control. Bacterial metabolic responses to fermentation, in addition to substrate type and availability, may also change as a function of the pH, since each bacterial group has an optimal pH level (Louis et al., 2007). In this sense, in *L. acidophilus* La-5 fermentation, the marked decrease in pH for FOS may be associated to the inhibition of butyrate production, resulting in similar values ($p>0.05$) to the negative control. Overall, our results indicate that the FVBP flour sample may have an interesting modulatory effect, as it allowed for the growth of potentially beneficial microorganisms while stimulating the production of butyrate.

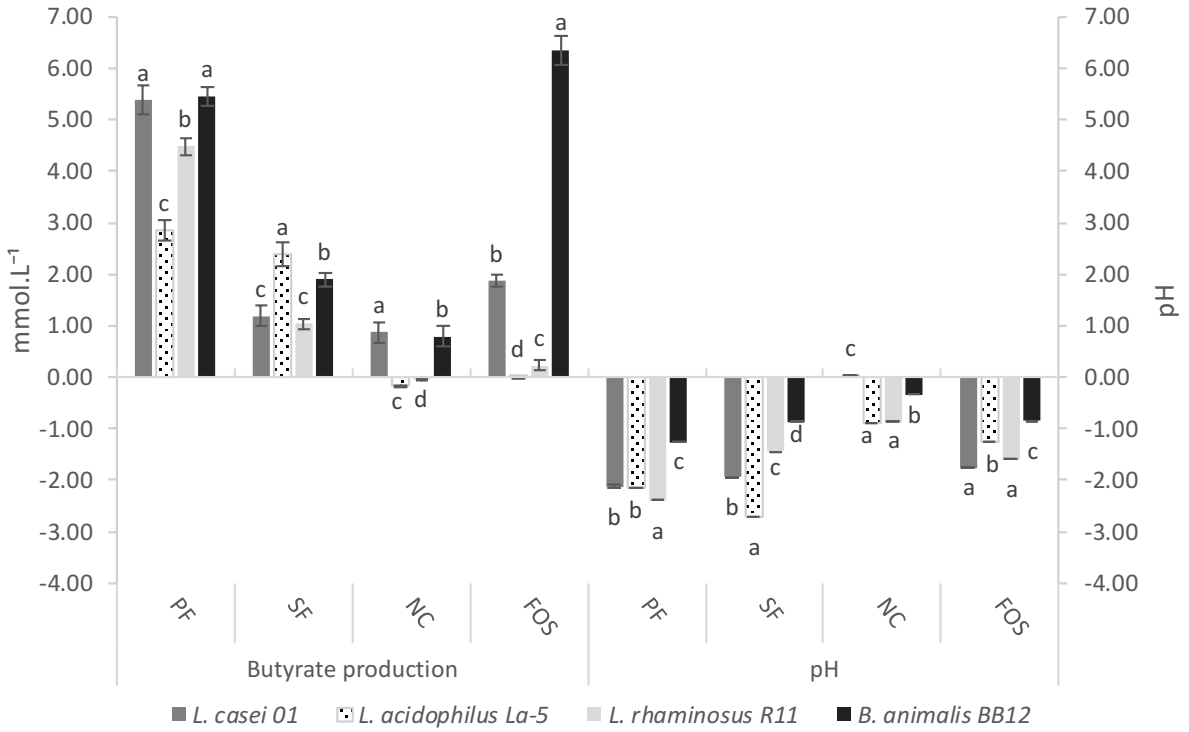

Figure 2. Butyrate production (mmol.L^{-1}) and pH values of four bacterial strains fermentation for 48 h with pellet fraction (PF), soluble fraction (SF), FOS and basal medium (negative control, NC). Values are expressed as mean ± standard deviation. For each sample, bars followed by the same lowercase letter are not significantly different by Tukey´s test ($p > 0.05$).

The results of potential prebiotic effect assay performed with donors fecal microbiota (Figure 3) demonstrated that, among the groups evaluated, the microbiota evaluated at 0 h fermentation time consisted mainly of the Firmicutes group (0.443 ± 0.021 log 16S rRNA gene copies/ng of DNA), followed by the *Clostridium leptum* subgroup (0.326 ± 0.021 log 16S rRNA gene copies/ng of DNA), Bacteroidetes (0.312 ± 0.025 log 16S rRNA gene copies/ng of DNA) and *Bacteroides* (0.295 ± 0.021 log 16S rRNA gene copies/ng of DNA), and with a lower proportion of *Bifidobacterium* (0.221 ± 0.019 log 16S rRNA gene copies/ng of DNA) and *Lactobacillus* (0.154 ± 0.030 log 16S rRNA gene copies/ng of DNA). These findings corroborated the literature, which shows that Firmicutes and Bacteroidetes are the primary phyla comprising healthy gut microbiota, constituting 80-90% of gut microbiota (Arboleya, Watkins, Stanton, & Ross, 2016). The most abundant species in the Firmicutes phylum is the *Clostridium leptum* subgroup, also called *Clostridium cluster* IV, accounting for 16- 25% of total gut microbiota (Louis et al., 2007; Sghir et al., 2000). *Bacteroides* represents the most abundant genera of the phylum Bacteroidetes and constitutes approximately 25% of

the gut microbiota (Parkar et al., 2018). In contrast, *Lactobacillus* (Firmicutes phylum) and *Bifidobacterium* (Actinobacteria phylum) are known to be beneficial to human health and have been the primary targets of prebiotic studies, but they only constitute approximately 2% and 4% of gut microbiota, respectively (Louis, Scott , Duncan & Flint, 2007).

Bacteria from Firmicutes and Bacteroidetes phylum possess the ability to break down plant cell wall oligo- and polysaccharides (Flint, Scott, Duncan, Louis, & Forano, 2012). In addition, species of the *Clostridium leptum* subgroup, such as those in the *Ruminococcus* group, can efficiently metabolize insoluble fibers such as lignocellulose (Ze, Le Mougen, Duncan, Louis, & Flint, 2013). Although FVBP flour contains a diversified and high content of lignocellulosic compounds, such as cellulose (19.1%) and insoluble lignin (14.9%) (T. B. Brito et al., 2019), it had no significant ($p>0.05$) influence on Firmicutes, Bacteroidetes, *Clostridium leptum* subgroup and *Bacteroides* growth during fermentation (Figure 3). The same result was observed for FOS. In this case, it is important to note the sensitivity of the microbiota to the acidic pH of the medium. *In vitro* fermentation models generally set pH in the medium as more acidic than that of the human colon. This can improve the growth of Firmicutes while decreasing the proliferation of *Bacteroides* (Holscher, 2017). In addition, researchers have suggested that high inter-donor variability may lead to statistically non-significant changes in response to the potential prebiotic, similar to those reported in other studies with human donor feces (Healey et al., 2017; Reichardt et al., 2018). Importantly, some species of the *Clostridium* and *Bacteroides* groups may harm host health; growth inhibition of these species may be beneficial (Pérez-López et al., 2016).

Interestingly, FVBP flour showed a greater potential prebiotic effect than FOS ($p <0.05$) on growth promotion of *Lactobacillus* after 48 h of fermentation, along with a similar bifidogenic effect to that of FOS on *Bifidobacterium* growth at 12, 24 and 48 h of fermentation. It is important to note that in our study, FOS had no effect on *Lactobacillus* growth at 12 and 24 h of fermentation, compared to the negative control.

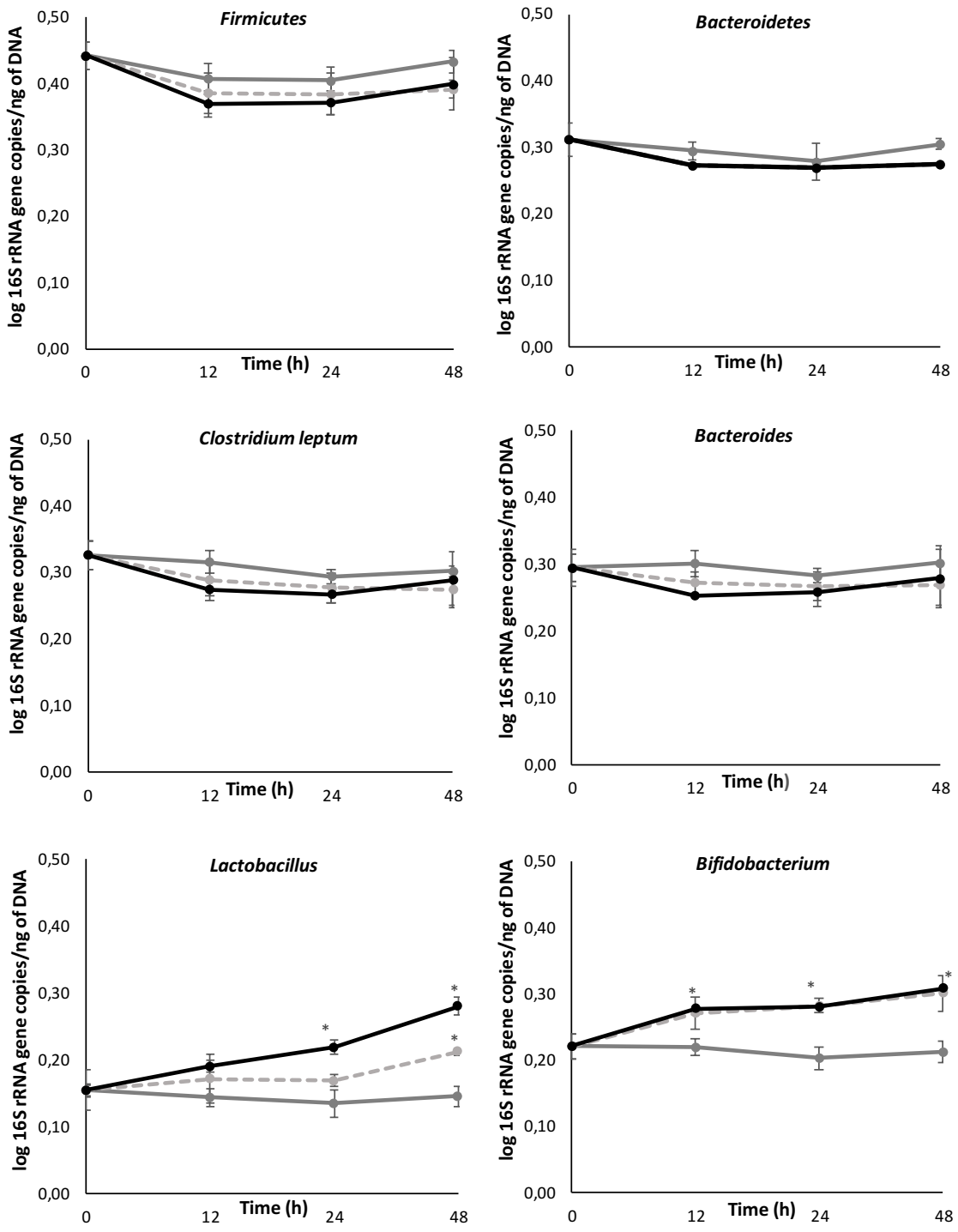

Figure 3. Six d*istinct bacterial groups variation of five donors microbiota. Values are express as log 16S rRNA gene copies/ng of DNA through time (h), with standard deviation bars.* ●—● *FVBP flour;* ●- - -● *FOS, positive control;* ●—● *C-, negative control.* For each bacterial group, the point of fermentation indicated by the sign (*) shows a significant difference for the negative control by Tukey´s test ($p > 0.05$).

The observed difference between FVBP flour and FOS in promoting *Lactobacillus* and *Bifidobacterium* growth may be explained as a function of these sample composition. In contrast to FOS, FVBP flour has a composition rich in dietary fiber (48%), in which the insoluble fraction is the most important: cellulose (19.1%), insoluble lignin (14.9%), hemicellulose (6.5%) and resistant starch (< 1%). Available carbohydrates constitute 26%, of which total starch represents 15%; there is also significant protein (9.5%) and soluble fiber (9.6%) content (Andrade et al., 2016; Brito et al., 2019; Ferreira et al., 2015). *Lactobacillus* shows high variability in carbohydrate metabolism among species; however, they may grow using a lower number of fermentation substrates compared to *Bifidobacterium* (Watson et al., 2013). In contrast, oligosaccharides are the preferred substrates for *Bifidobacterium* fermentation. However, this group may also ferment polysaccharides, because most of its species express glycoside hydrolase, the most important enzyme group to degrade poly- and oligosaccharides (Van Den Broek & Voragen, 2008). Thus, we suggest that *Bifidobacterium* may commence the FVBP flour and FOS fermentation processes, which could explain the faster effect on its growth. Subsequently, for FVBP flour after 24h of fermentation, *Lactobacillus* growth stimulation occurs through the cross-feeding effect.

Regarding the non-influence of FOS on *Lactobacillus* growth, researchers have described that, under controlled conditions with FOS as the carbon source, *Lactobacillus* can compete with *Bifidobacterium* (Sghir et al., 1998). Moreover, *Lactobacillus* requires amino acids, peptides, and other compounds in addition to non-digestible carbohydrates for growth (Sghir et al., 1998)—nutrients found in the FVBP flour matrix. As well as a rich dietary fiber composition, FVBP also presented a diversified phenolic profile after pressurized liquid extraction and UPLC-MS-MS characterization (Gonçalves et al., 2018). The fiber-bound polyphenols in the food matrix can reach the colon, where they may be metabolized by the gut microbiota and contribute to the health-related properties attributed to dietary fiber and its impact on gut microbiota modulation (Pérez-Jiménez et al., 2013; Tomás-Barberán et al., 2016).

The acetate, lactate, propionate, and butyrate production were evaluated at 0, 12, 24, and 48 h of fermentation for FVBP flour and control samples (Table 3). At time 0 h, the production of organic acids was not detected. Acetate and lactate content for FOS and FVBP flour fermentation was higher ($p<0.05$) than that of propionate and butyrate at all times evaluated. *Bifidobacterium* and heterofermentative *Lactobacillus* are lactic acid bacteria that produce acetate and lactate as fermentation end products (Holscher, 2017). In addition,

Bifidobacterium can produce acetate and lactate from carbohydrate substrates in molar proportions of 3: 2 (Duncan et al., 2004), a proportion similar to that found in our study using FVBP flour as a carbon source. However, despite an increase of acetate content after 24h of fermentation for all samples ($p<0.05$), the values for FVBP flour showed no significant difference to the control samples ($p>0.05$) during *in vitro* fermentation. According to Hernández et al. (2019), the configuration of the glycosidic bonds of dietary fiber and the fermentation delay in fiber mixture samples can affect the production of acetate by the gut microbiota. In this sense, we suggest that the complex mixture of FVBP flour matrix, with a higher and varied content of lignocellulosic compounds and significant content of starchy carbohydrates, including resistant starch, may have decreased the acetate production when FVBP flour was used as carbon source. In addition, according to Tuncil et al. (2017), the effects of dietary fibers on the colonic microbiota are highly dependent on the initial microbiota community of individuals.

The lactate content for FVBP flour remained stable and similar to FOS during *in vitro* fermentation ($p>0.05$) while a significant increase in lactate content ($p<0.05$) for FOS was observed at 48 h. It is known that butyrate-producing colon bacteria, as certain Firmicutes groups, can convert acetate and lactate to butyrate, which normally lowers lactate levels in adult fecal samples (Flint 2012; Flint 2014). However, Belenguer et al. (2007) reported that in fecal inocula with mix of polysaccharides at lower pH, the use of lactate is reduced while its production is maintained, resulting in lactate accumulation. Thus, we suggest that the lactate accumulation observed in our study with FOS and FVBP flour may result from changes in pH during *in vitro* fermentation.

The propionate content for FVBP flour showed no difference from the control samples until 24 h and was lower than the negative control ($p<0.05$) at 48 h. At this time, the negative control showed a slight increase in *Bacteroides* growth, which are known to be able to produce propionate (Gómez et al., 2014). In contrast, there was no influence on *Bacteroides* growth for FVBP flour and FOS during *in vitro* fermentation. This finding can be explained by the deep decrease in pH of these samples during *in vitro* fermentation, which was not observed for negative control sample. The propionate production is related to the proportion of propionate-producing bacteria and, under acidic conditions, there is a reduction in acid-sensitive bacteria growth such as *Bacteroides* spp., that represent the main propionate producers in the gut (Holscher, 2017; Reichardt et al., 2014, 2018).

Table 3. Concentrations of short chain fatty acids (SCFA) and lactate produced and pH values during fermentation of FVBP flour, FOS and negative control (C).

Time (h)	Sample	Acetate (mg.mL^{-1})	Lactate (mg.mL^{-1})	Propionate (mg.mL^{-1})	Butyrate (mg.mL^{-1})	Total SCFAs and Lactate (mg.mL^{-1})	pH
12	C-	0.175 ± 0.036c	nd	0.067 ± 0.004ab	nd	0.242 ± 0.036c	5.88 ± 0.13b
	FOS	0.209 ± 0.070bc	0.130 ± 0.051b	nd	nd	0.339 ± 0.070c	4.20 ± 0.21cd
	FVBP	0.276 ± 0.044bc	0.119 ± 0.042b	0.053 ± 0.002b	nd	0.448 ± 0.035bc	4.61 ± 0.30c
24	C-	0.507 ± 0.093a	nd	0.064 ± 0.009ab	0.012 ± 0.001b	0.583 ± 0.095b	6.29 ± 0.08ab
	FOS	0.477 ± 0.072a	0.172 ± 0.039b	nq	0.045 ± 0.012a	0.694 ± 0.077ab	3.77 ± 0.27de
	FVBP	0.367 ± 0.050ab	0.148 ± 0.053b	0.055 ± 0.002b	0.028 ± 0.009a	0.598 ± 0.042b	4.61 ± 0.32c
48	C-	0.457 ± 0.091a	nd	0.082 ± 0.018a	0.024 ± 0.007ab	0.563 ± 0.109b	6.40 ± 0.16a
	FOS	0.329 ± 0.081abc	0.319 ± 0.088a	0.056 ± 0.015b	0.018 ± 0.004b	0.723 ± 0.089a	3.49 ± 0.18e
	FVBP	0.455 ± 0.043a	0.144 ± 0.024b	0.054 ± 0.009b	0.026 ± 0.006a	0.679 ± 0.039ab	4.39 ± 0.21c

nd: not detected. Values are expressed as mean ± standard deviation. Values followed by the same lowercase letter within the same column are not significantly different by Tukey´s test ($p>0.05$).

Regarding butyrate, FOS and FVBP flour both showed higher butyrate content than negative control at 24 h, with FOS having the highest levels ($p > 0.05$). It is important to note that although we did not observed an increase in butyrate-producing bacteria, such as the Firmicutes and *Clostridium* groups, we did verify a butyrogenic effect from FVBP flour and FOS as carbon sources at 24 h. This can be explained by the ability of Firmicutes species to convert lactate and acetate into butyrate, through cross-feeding interactions (Flint et al., 2012; Rivière et al., 2016). Summary, we suggest that the configuration of the fiber-rich FVBP flour matrix and a deep decrease in pH during *in vitro* fermentation may have influenced the *Bifidobacterium* and *Lactobacillus* growth and the inhibition of the propionate- and butyrate-producing bacteria growth. Similarly, Liu et al., (2017) reported that intervention with high-dose of prebiotics mainly promoted the proliferation of *Bifidobacterium* and the production of much lactic acid but hindering the growth of butyrate-producing and pathogenic bacteria and the production of SCFAs. However, it is important to note that the results of our study demonstrate that the metabolic pathway through the FVBP flour fermentation is not elucidated and further studies on the effect of the structure of a fiber-rich matrix on the production of SCFA by the gut microbiota must be carried out, including pathogenic bacteria.

The evaluation of sample pH showed that in all fermentation times evaluated, media with FOS and FVBP flour samples had lower pH values ($p<0.05$) than negative control sample (Table 3). The reduction in pH during fermentation of non-digestible carbohydrate sources is well-described in the literature (Duncan et al., 2009; Gómez et al., 2016). The decrease in pH may promote butyrate production by butyrate-producing bacteria (Reichardt et al., 2018), but also can inhibit the growth of pathogenic bacteria (Cantu-Jungles et al., 2017).

Figure 4 illustrates FVBP flour cytotoxicity analysis (at 3 and 6% concentrations), before and after digestion, on an intestinal Caco-2 cell line. The Caco-2 cell line, derived from human colon carcinoma, has characteristics similar to enterocytes, being a viable cell culture model to mimic the intestinal epithelium in *in vitro* studies (Artursson & Karlsson, 1991). Thus, we observed that FVBP flour at 3% concentration, after *in vitro* gastrointestinal digestion, increased cell metabolism up to 67%. In contrast, before digestion, FVBP flour at the same concentration significantly inhibited cell metabolism by 53%. We posit that a reduced impact on cell metabolism after digestion occurs due to the release of antioxidant compounds, such as polyphenols, from the complex matrix of fruit and vegetable byproducts during gastrointestinal digestion. In agreement, the antioxidant capacity of FVBP flour was assessed during *in vitro* digestion and showed a significant increase after the intestine step by

the ABTS and ORAC assays (data not shown). Similarly, Cilla et al. (2008) reported in a study with fruit beverages that a cytoprotective effect on Caco-2 cells is derived from bioaccessible fractions of the beverages. The protective effect of natural antioxidants like polyphenols and carotenoids against oxidative damage to Caco-2 cell lines (Cilla et al., 2008; Ramos et al., 2010; S. Wang et al., 2016) can promote metabolism in this cell line.

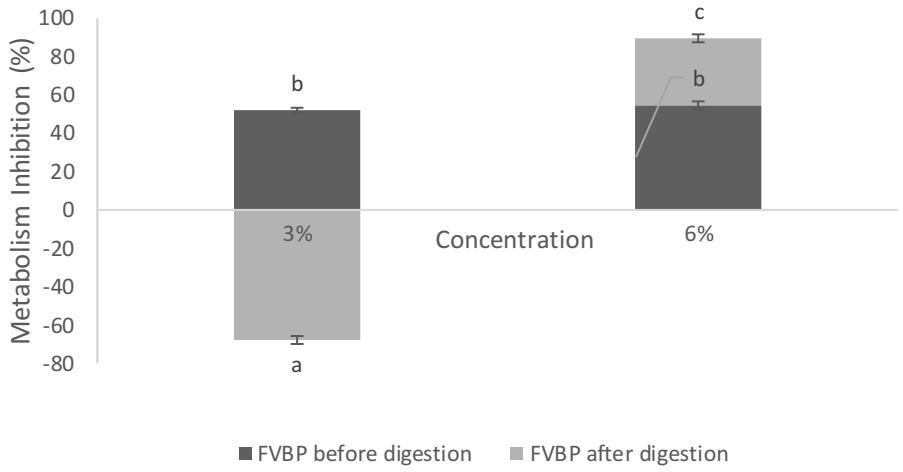

Figure 4. Metabolism Inhibition (%) of FVBP flour at concentrations of 3% and 6% (w/v) before and after *in vitro* digestion on metabolism of Caucasian colon adenocarcinoma cell (Caco-2 cell). Bars fractions followed by the same lowercase letter are not significantly different by Tukey´s test ($p > 0.05$).

Similarly, after digestion, the sample with the higher concentration of FVBP flour (6%) demonstrated inhibition of cell metabolism significantly lower than before digestion ($p < 0.05$), but both still negatively impacted cell metabolism of 35% and 51%, respectively. In this regard, we suggest that during digestion of FVBP flour at 6% concentration, the high levels of antioxidants and unmeasured digestion metabolites may be associated with the significant inhibition of cell metabolism observed. We recognize that antioxidant compounds may exert a pro-oxidant activity *in vitro*, especially in the presence of metal ions (Cilla et al., 2008; Eghbaliferiz & Iranshahi, 2016). Therefore, further studies are needed to evaluate changes in food matrix structures during digestion along with the interactions and impact of all digestion metabolites on intestinal epithelium. Furthermore, it is important to emphasize that the cells were in direct contact with the samples tested, although they were diluted in culture media. In an *in vivo* situation, this exposure rarely occurs in such a direct way and, as such, may also have contributed to the inhibition values registered.

4. Conclusion

The present study describes the prebiotic potential of fruit and vegetable by-product flour after *in vitro* gastrointestinal digestion. It is important to note that no chemical or enzymatic extraction methods were used to obtain this flour, which demonstrates the viability of its use in a sustainable food system at a low cost. After digestion, it was verified that FVBP flour demonstrated a positive effect on *Bifidobacterium* and *Lactobacillus* growth in donor feces, equal to or better than FOS. It is suggested that the composition of FVBP flour, high in insoluble fiber, especially composed by lignocellulosic compounds and available carbohydrates (mono- and disaccharides), in addition to a significant protein fraction and soluble fiber may be associated with this effect. Furthermore, even with donor inter-individual variability and limitations of *in vitro* model, it was possible to identify the short chain fatty acid production with FVBP flour as carbon source, including butyrate after 24 h of fermentation. These results demonstrate the feasibility of low-cost flour produced from discarded fruit and vegetables representing a sustainable food/ingredient with potential prebiotic activity.

Acknowledgements / Funding

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5. CONCLUSÃO GERAL

O presente trabalho avaliou o impacto da digestão gastrointestinal nas propriedades funcionais de uma farinha produzida a partir de subprodutos do processamento de frutas e hortaliças (FVBP). A bioacessibilidade dos compostos bioativos, as propriedades antioxidantes e prebiótica deste subproduto foram determinadas após digestão gastrointestinal *in vitro*. Com os resultados obtidos foi possível identificar um efeito positivo da digestão gastrointestinal na capacidade antioxidante e na bioacessibilidade de carotenoides e aminoácidos da farinha FVBP. Foi verificado um aumento no conteúdo de todos os aminoácidos ao longo da digestão, com maiores concentrações de glutamina e arginina após a etapa intestinal, que podem promover o trofismo da mucosa intestinal. O conteúdo total de carotenoides permaneceu estável durante a digestão, no entanto, observou-se um ligeiro aumento na fase gástrica da digestão, o que pode estar relacionado à redução do pH.

A bioacessibilidade dos compostos fenólicos foi de 38% e houve uma redução no índice de recuperação destes compostos após a fase intestinal, apesar do aumento simultâneo da capacidade antioxidante da fração solúvel da farinha FVBP. A capacidade antioxidante aumentou após as etapas gástrica e intestinal da digestão, o que sugere elevada liberação de compostos antioxidantes da matriz da farinha, durante a digestão. O perfil de polifenóis também foi modificado, principalmente quanto ao aumento no teor de flavonoides após a digestão, porém, um impacto negativo foi observado sobre o teor de ácidos fenólicos. Sugere-se que os compostos não liberados ou não detectados após a etapa intestinal possam estar retidos no resíduo não digerido e, assim, atingem o cólon, onde serão liberados pela ação da microbiota intestinal promovendo efeitos benéficos à saúde intestinal.

A determinação do potencial prebiótico da farinha FVBP demonstrou efeito positivo sobre o crescimento de *Bifidobacterium* e *Lactobacillus* nas fezes dos doadores, semelhante ou até melhor que a amostra de controle de positivo FOS. O efeito positivo da farinha pode estar relacionado ao elevado teor de fibra insolúvel e carboidratos simples, além de teor significativo de fibra solúvel e proteína. O potencial prebiótico foi corroborado pela produção de ácidos graxos de cadeia curta durante a fermentação fecal com a farinha FVBP usada como fonte de carbono. A produção de butirato após 24 h de fermentação de FOS e FVBP foi superior ao controle negativo. Entretanto, a produção de acetato e lactato com fermentação de FOS e FVBP foi maior ($p < 0,05$) do que a de propionato e butirato em todos os períodos avaliados.

Os resultados obtidos no presente estudo demonstram as propriedades funcionais da farinha desenvolvida a partir de subprodutos do processamento de frutas e hortaliças como o aumento na capacidade antioxidante e no teor de flavonoides, aminoácidos e estabilidade de carotenoides após a digestão gastrointestinal, além do potencial prebiótico determinado. Além disso, sugere-se que o potencial prebiótico observado na farinha FVBP esteja relacionado a ação conjunta de compostos presentes, pincipalmente oligo- e polissacarídeos, além de mono- e dissacarídeos e polifenóis e seus metabólitos, mas com possível ação de peptídeos.

Desta forma, estudos adicionais são necessários para investigar a estrutura e a interação de todos os compostos da matriz de subprodutos alimentares, antes e após a digestão gastrointestinal e para explorar o impacto de cada composto e seus metabólitos na modulação da microbiota intestinal. Além disso, estudos *in vivo* para avaliação do impacto de compostos funcionais e bioativos após a digestão sobre a microbiota intestinal são necessários para corroborar os achados *in vitro* do presente estudo. Tais estudos podem estender o uso de subprodutos como ingredientes alimentares funcionais e como fonte de compostos funcionais.

A farinha de subprodutos de frutas e hortaliças mostrou ser uma alternativa viável para utilização dos resíduos gerados no processamento de vegetais, principalmente no que tange a bioacessibilidade de compostos bioativos. É importante destacar que não foram utilizadas reações químicas ou enzimáticas ou tecnologias de custo elevado para obtenção desta farinha, o que corrobora com a viabilidade do uso desta matéria-prima em uma cadeia de alimentos sustentável a um baixo-custo. Além disso, a farinha representa um ingrediente com propriedades funcionais, incluindo potencial prebiótico e, dessa forma, sugere-se denominá-la como alimento/ingrediente prebiótico sustentável.

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