INSTITUTO DE TECNOLOGIA DE ALIMENTOS

Centro de Ciência e Qualidade de Alimentos

MARIANA SISCONETO BISINOTTO

PROSPECÇÃO DA CAPACIDADE ANTIOXIDANTE E POTENCIAL EFEITO PREBIÓTICO DE COMPOSTOS LIBERADOS APÓS DIGESTÃO *IN VITRO* DE FARINHAS DESENGORDURADAS DE GIRASSOL, DE PALMISTE E DE CASTANHA-DE-CAJU

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RESUMO

A valorização de co-produtos agroindustriais surge como uma tendência mundial no contexto da sustentabilidade, como meio de tornar o agronegócio mais rentável, aumentar a oferta de alimentos no mundo e extrair compostos bioativos benéficos à saúde. Os co-produtos resultante da extração do óleo vegetal, as farinhas desengorduradas de palmiste (FDP), girassol (FDG) e castanha-de-caju (FDC) foram estudadas nesse trabalho como potencial fonte de proteínas vegetais, fibras dietéticas e compostos fenólicos capazes de desenvolver capacidade antioxidante e potencial efeito prebiótico após a digestão simulada. Dessa forma, este estudo teve por objetivo avaliar o efeito da digestão gastrointestinal *in vitro* (DGI) na bioacessibilidade de proteínas e fenólicos, bem como avaliar seu impacto na capacidade antioxidante e potencial efeito prebiótico. A digestão consistiu-se da fase oral (10 min, em pH 7), gástrica e intestinal, ambas conduzidas por 2 h cada, em pH 3 e 7, respectivamente. Toda DGI foi realizada a 37°C, sob agitação de 90 rpm. Para interromper a DGI, o volume total foi aquecido a 90°C / 10 min e resfriado a 4°C. As frações, solúvel (Ds) e insolúvel (Di), foram separadas por centrifugação (3645 x g / 30 min/ 4°C) e liofilizadas. Estudos preliminares mostraram a necessidade de realizar um pré-tratamento na FDP para aumentar sua solubilidade. Todas as matérias-primas tiveram sua composição físico-química determinada. Nas amostras de FDG, FDP, FDC e suas respectivas frações solúveis (Ds-G, Ds-P e Ds-C) foi determinado o perfil de aminoácidos totais e a distribuição de peso molecular (PM) por SE-FPLC. Extratos aquosos e hidroalcóolicos foram feitos para determinar compostos fenólicos totais por reagente de Folin-Ciocalteau e capacidade antioxidante pelos métodos de ORAC, ABTS, DPPH e proteção ao DNA. As frações insolúveis (Di) dos digeridos de FDP (Di-P), FDG (Di-G) e FDC (Di-C) foram utilizadas como fonte de carbono em meio de cultura MRS, nos quais inoculou-se cepas de bactérias probióticas comerciais (*Lactobacillus acidophilus, Lactiplantibacillus plantarum* e *Bifidobacterium animalis*). A distribuição de PM mostrou que 83,24%, 73,47% e 35,06% dos compostos proteicos na Ds-C, Ds-G e Ds-P apresentam PM no intervalor de 0,1 kDa <PM< 3 kDa, importante para exercer bioatividades. O escore químico das proteínas se mostrou adequado para as farinhas e a DGI aumentou a solubilidade de aminoácidos antioxidantes (como

tirosina e triptofano). As Ds-G, Ds-P e Ds-C apresentaram maior teor de compostos fenólicos e capacidade antioxidante que as respectivas farinhas em todas as análises. A Di-C ocasionou crescimento superior aos tratamentos controles de *Bifidobacterium lactis* BB-12, possuindo carboidratos não digeríveis passíveis de serem fermentados por esta bactéria. Concluindo, a DGI liberou compostos bioativos, como peptídeos e compostos fenólicos, que apresentam capacidade antioxidante e a fração Di-C apresentou potencial efeito prebiótico.

Palavras-chave:

Helianthus annuus L.; *Elaeis guineensis Jacq.*; *Anacardium occidentale L.*;
digestão *in vitro;* antioxidante.

ABSTRACT

Agroindustry by-products valorization rises as a worldwide trend in the sustainability context, an attempted to increase agribusiness profitability, the food supply worldwide and to extract biocomponents that have health benefits. The co-products generated through vegetable oil extraction, as defatted flours of palm kernel (FDP), sunflower (FDG) and cashew nut kernel (FDC) were studied as plant proteins, dietary fibers and phenolic compounds potential source, capable of antioxidant capacity and potential prebiotic effect development. This study aimed to evaluate the *in vitro* gastrointestinal digestion (DGI) impact on the plant proteins and phenolic compounds bioaccessibility, as well its impact on the antioxidant capacity and potential prebiotic effect. The digestion was consisted of an oral phase (10 min, at pH 7), gastric and intestinal phases, both during 2 h each, at pH 3 and 7, respectively. All the DGI was accomplished at 37 °C, under 90 rpm stirring. For DGI interruption, the whole volume was heated up to 90°C / 10 min and then cooled-down to 4°C. The soluble (Ds) and insoluble (Di) fractions were separated by centrifugation (3645 x g / 30 min/ 4°C), and then freeze-dried. Preliminary studies have shown the necessity to pretreat the FDP in order to increase its solubility. All raw materials have had their physicochemical composition determined. Samples of FDG, FDP, FDC and their respective soluble fractions (Ds-G, Ds-P and Ds-C) were used to determine total amino acid profile and molecular weight (MW) distribution by SE-FPLC. Aqueous and hydroalcoholic extracts were made aiming to determine total phenolics compounds by Folin-Ciocalteau reagent and antioxidant capacity through the ORAC, ABTS, DPPH and DNA protection methods. The insoluble fractions of FDP (Di-P), DFG (Di-G) and FDC (Di-C) were used as carbon source in MRS media, where commercial strains of probiotic bacteria were inoculated (*Lactobacillus acidophilus, Lactiplantibacillus plantarum* and *Bifidobacterium animalis*). The MW distribution showed that 83.24%, 73.47% and 35.06% of Ds-C, Ds-G and Ds-P soluble compounds had MW from 0.1kDa to 3kDa, which is important to develop bioactivities. The protein's amino acid scores were adequate and the DGI increased the solubility of antioxidant amino acid (such as tyrosine and tryptophan). The Ds-G, Ds-P and Ds-C fractions showed higher phenolic compounds and antioxidant capacity compared to their respective flour in all assays.

The Di-C lead to a *Bifidobacterium lactis* BB-12 growth higher than the negative control, suggesting the presence of non-digested carbohydrates which could be fermented by the bacteria. In conclusion, the DGI released bioactive compounds, as peptides and phenolic compounds which showed antioxidant capacity and the Di-C fraction showed potential prebiotic effect.

Key words:

Helianthus annuus L.; *Elaeis guineensis Jacq.*; *Anacardium occidentale L.*; *in vitro* digestion; antioxidant.

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LISTA DE ABREVIATURAS E SIGLAS

ABTS: 2,2-azino-bis (3-etilbenzotiazolin)-6-sulfônico

AAPH: 2,2'-azobis(2-amidino-propano) dihidroclorado

DCNT: doenças crônicas não-transmissíveis

DGI: digestão gastrointestinal simulada *in vitro*

DPPH: 2,2 difenil-1-pricril-hidrazil

Ds: fração solúvel do digerido gastrointestinal de farinha desengordurada

Ds-C: fração solúvel do digerido gastrointestinal de farinha desengordurada de castanha-de-caju

Ds-G: fração solúvel do digerido gastrointestinal de farinha desengordurada de girassol

Ds-P: fração solúvel do digerido gastrointestinal de farinha desengordurada de palmiste

Di: fração insolúvel do digerido gastrointestinal de farinha desengordurada

Di-C: fração insolúvel do digerido gastrointestinal de farinha desengordurada de castanha-de-caju

Di-G: fração insolúvel do digerido gastrointestinal de farinha desengordurada de girassol

Di-P: fração insolúvel do digerido gastrointestinal de farinha desengordurada de palmiste

ERN: espécies reativas de nitrogênio

ERO: espécies reativas de oxigênio

FDC: farinha desengordurada de castanha-de-caju

FDG: farinha desengordurada de girassol

FDP: farinha desengordurada de palmiste

MOS: manano-oligosacarídeos

MW: molecular weight

ORAC: Oxigen radical absortion capacity / capacidade de absorção de radical oxigênio

PKC: palm kernel cake / torta de palmiste

PM: peso molecular

$ROO^{\bullet}$: radical peroxil

SIF: simulated intestinal fluid

SGF: simulated gastric fluid

SSF: simulated salivary fluid

INTRODUÇÃO

Vivemos em um cenário globalmente fragilizado, em função do acelerado e desregrado consumo dos recursos naturais renováveis e não renováveis, do crescimento da população mundial acometida de profundos problemas sociais e da constante pressão econômica. Assim, a sustentabilidade tem se consolidado como uma tendência global em resposta aos anseios mundiais de equilibrar as esferas ambiental, econômica e social. (GEISSDOERFER et al., 2017). Diversas ações têm sido realizadas nesse contexto, como a adoção do modelo de economia circular e a valorização de co-produtos agroindustriais, objetivando reduzir o impacto ambiental e tornar o agro negócio mais rentável. (CONTRERAS et al., 2019; MURRAY; SKENE; HAYNES, 2017).

Os co-produtos da agroindústria apresentam em sua composição componentes de elevado valor biológico como polifenóis, polissacarídeos e peptídeos que tem sido estudados extensivamente quanto ao benefício à saúde e propriedades anti-inflamatória, antimicrobiana, antioxidante e anticarcinogênica (GULLÓN et al., 2020). Uma vez extraídos, podem ser reutilizados como ingredientes alimentares com funções nutricionais e bioativas, além de serem aplicados em formulações farmacológicas (ALEXANDRE et al., 2018).

Fazer uso da dieta para controlar desordens de saúde e modular a microbiota intestinal é uma abordagem comum em ciências nutricionais além de atrair consumidores (VIEIRA DA SILVA; BARREIRA; OLIVEIRA, 2016). Além disso existe uma acentuada demanda por produtos proteicos de origem vegetal, em função do crescimento dos públicos vegetariano e veganos, principalmente nos países mais desenvolvidos (HAYES, 2018). As proteínas vegetais de oleaginosas apresentam elevado valor biológico em função da presença de aminoácidos essenciais e tem sido utilizadas para suplementar a dieta humana, frequentemente aplicada na fortificação de produtos de panificação a base de cereais (LAI et al., 2017). Proteínas de origem animal e vegetal são fontes potenciais de uma vasta quantidade de peptídeos bioativos, presentes em suas estruturas originais, e que ao serem liberados podem exercer diversas bioatividades, por exemplo a antioxidantes (SÁNCHEZ; VÁZQUEZ, 2017). Compostos fenólicos são metabólitos secundários de plantas, antioxidantes naturais (VIEIRA DA SILVA; BARREIRA; OLIVEIRA, 2016) e o consumo de alimentos com alta concentração desse composto tem sido correlacionado à benefícios à saúde, apresentando propriedades anti-câncer, anti-microbiana e anti-inflamatória (SANCHIZ et al., 2019). Os antioxidantes ganham destaques ao contribuírem

para prevenção do estresse oxidativo, que apresenta correlação com o desenvolvimento de doenças crônicas não-transmissíveis (DCNT) (ZHANG; MU; SUN, 2014). Estudos recentes revelam também a importância da composição da microbiota intestinal e sua correlação com a saúde geral do hospedeiro, sendo relevante prospectar o efeito prebiótico de alimentos. As fibras, constituinte dos carboidratos que compões o farelo desengordurado, podem ser aplicados na fabricação de produtos de panificação com maior teor de fibras (LAI et al., 2017).

A presente pesquisa teve como foco o aproveitamento de tortas desengorduradas de oleaginosas, biomassa resultante da fabricação de óleo vegetal. A partir de tortas de girassol, palmiste e aparas de castanha-de-caju foram obtidas farinhas desengorduradas. Realizou-se a digestão gastrointestinal *in vitro* para avaliar a capacidade antioxidante e o potencial efeito prebiótico. A hipótese do trabalho é que a digestão simulada é capaz de aumentar a solubilidade de proteínas e de compostos fenólicos, sendo passíveis de serem absorvidos para exercer a bioatividade antioxidante enquanto os carboidratos não-digeríveis podem ser fermentados por bactérias probióticas, exercendo assim um potencial efeito prebiótico. Fazendo-se uso integral das farinhas, ao invés de extrair seus compostos bioativos isolados, objetivou-se avaliar duas bioatividades com o menor emprego de processos industriais para o aproveitamento desses co-produtos, além de evitar a produção de outros resíduos.

Esta dissertação de mestrado foi estruturada no formato de artigo. O capítulo 1 apresenta uma revisão bibliográfica sobre o assunto abordado. Já os capítulos 2 e 3 trazem os artigos elaborados a partir dos resultados obtidos. No capítulo 2, o artigo foi redigido em conformidade às normas da revista Food Research International, abordando a farinha desengordurada de castanha-de-caju. Por sua vez, no capítulo 3, o artigo foi redigido de acordo com as normas da revista Food Chemistry, abordando as farinhas desengorduradas de girassol e palmiste. Essa distinção foi feita em função da natureza das matrizes estudadas, que apesar de serem todas culturas oleaginosas, se dividem entre as categorias de noz (castanha-de-caju) e sementes (girassol e palmiste).

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OBJETIVOS

OBJETIVO PRINCIPAL

Avaliar a capacidade antioxidante das frações solúveis e potencial efeito prebiótico das frações insolúveis dos digeridos gastrointestinais de farinhas desengorduradas de girassol, palmiste e castanha-de-caju.

OBJETIVOS ESPECÍFICOS

Extrair o óleo residual dos farelos de girassol, palmiste e castanha-de-caju com n-hexano para obter as respectivas farinhas desengorduradas;

Realizar digestão gastrointestinal *in vitro* dessas farinhas, separar as frações solúvel e insolúvel por centrifugação;

Caracterizar as farinhas e os digeridos gastrointestinais;

Determinar atividade antioxidante *in vitro* por ABTS, DPPH, ORAC e de proteção ao DNA das farinhas e de suas frações solúveis;

Prospectar efeito prebiótico das frações digeridas insolúvel em meio de cultura MRS com inoculação de bactérias probióticas comerciais.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1. Sustentabilidade: uma tendência mundial

A Revolução Industrial criou um sistema econômico linear extrativista, caracterizado por um intenso consumo dos recursos naturais renováveis e não renováveis como matéria-prima dos processos produtivos (LIEDER; RASHID, 2016). Consequentemente, são descartados no meio ambiente uma grande quantidade de poluentes e resíduos sólidos, gerados pelos processos produtivos, que ocasionaram importantes mudanças climáticas e danos a ecossistemas (MURRAY; SKENE; HAYNES, 2017). Tal situação foi agravada pelo crescimento da população mundial, acometida por graves problemas sociais, e por sistemas econômicos instáveis, instaurando-se assim um cenário socioeconômico globalmente fragilizado (GEISSDOERFER et al., 2017). Tal cenário suscitou diversas discussões internacionais que abordaram de forma holística temas como meio-ambiente, sociedade, economia e suas interconexões (GEISSDOERFER et al., 2017).

A comissão das Organização das Nações Unidas (ONU) realizada em 1987 resultou na elaboração do Relatório Brundtland, que define desenvolvimento sustentável como “aquele que atende às necessidades do presente sem comprometer a possibilidade de as gerações futuras atenderem às suas necessidades” (ONUBR, 2018). Diversas ações globais têm sido realizadas para implantar o desenvolvimento sustentável. Alguns exemplos são a Agenda 2030 para o Desenvolvimento Sustentável, elaborada pela ONU em 2015 que contempla 17 objetivos globais a serem incorporados em políticas e estratégias dos países membro (ONU, 2015) e o incentivo da implantação da economia circular no mundo (ELLEN MACARTHUR FOUNDATION, 2018).

A economia circular é um modelo de negócio pelo qual se espera equilibrar o desenvolvimento industrial, a conservação ambiental e a promoção da saúde humana (GHISELLINI; CIALANI; ULGIATI, 2016). Tal modelo popularizou-se na China nos anos 90 em resposta às consequências do rápido crescimento do país e tornou-se uma tendência mundial, com a publicação de diretrizes políticas em diversos países. Alguns exemplos são o novo “Pacote Europeu para a economia circular” (criado em 2020), a Lei de Economia Circular Chinesa (promulgada em 2016) e o surgimento de ONGs (a exemplo da Fundação Ellen MacArthur) que, associada a indústrias, à Academia e órgãos governamentais pretende implementar o conceito no mundo ocidental (GEISSDOERFER et al., 2017;

WINANS; KENDALL; DENG, 2017, UE, 2020). No Brasil, a Comissão Nacional da Indústria (CNI) elaborou um documento que incentiva a adoção da economia circular, afirmando ser uma decisão estratégica que permite à empresa compreender e redefinir sua atuação no setor ao qual está inserida (CNI, 2018).

O modelo de economia circular, em oposição ao sistema linear extrativista, propõe reconstruir o capital (seja este financeiro, de manufatura, humano, social ou natural) pela otimização dos fluxos de matérias primas e serviços, empregando-os em um ciclo fechado, regenerativo e contínuo em cada elo da cadeia de valor (ELLEN MACARTHUR FOUNDATION, 2018). São incorporados muitos outros conceitos na economia circular como o 3Rs (reduzir, reutilizar, reciclar) e 6Rs (reduzir, reutilizar, reciclar, recuperar, repensar e remanufaturar) (WINANS; KENDALL; DENG, 2017). São estabelecidos dois ciclos: o biogeoquímico e o de reciclagem de produtos. Pelo primeiro entende-se restaurar os ciclos naturais do planeta com a redução ou gerenciamento da retirada excessiva de matéria-prima e de retorno de resíduos ao meio-ambiente; pelo segundo prevê utilizar resíduos como recursos para novos ciclos produtivos, reduzindo-se o ritmo de consumo (com melhores técnicas de produção e manutenção dos produtos e serviços) e de descarte, que se torna matéria-prima para ciclos de produção subsequentes (MURRAY; SKENE; HAYNES, 2017). A Figura 1 demonstra esquematicamente o funcionamento da economia circular.

Segundo a última previsão feita pela Organização das Nações Unidas para Alimentos e Agricultura, FAO, estima-se que no ano de 2050 a população mundial será de 9,73 bilhões de habitantes e cerca de 11,2 bilhões em 2100 (FAO, 2017). Atualmente muitos são os desafios que a sociedade precisa enfrentar para garantir o fornecimento e distribuição de alimentos, mitigando os efeitos das mudanças climáticas e reduzindo a dependência de combustíveis fósseis (CONTRERAS et al., 2019). O estudo publicado pela FAO em 2011 ainda é a referência global para a cadeia de suprimentos de alimentos e estima que um terço dos alimentos produzidos para consumo humano no mundo tenha sido perdido ou desperdiçado, desde a produção agrícola até o consumo, equivalendo aproximadamente a 1,3 bilhões de toneladas de alimentos por ano (FAO, 2019).

Princípio 1
Preservar e aprimorar o capital natural controlando estoques finitos e equilibrando os fluxos de recursos renováveis.

Princípio 2
Otimizar o rendimento de recursos fazendo circular produtos, componentes e materiais em uso no mais alto nível de utilidade o tempo todo, tanto no ciclo técnico quanto no biológico.

Princípio 3
Estimular a efetividade do sistema revelando e excluindo as externalidades negativas desde o princípio.

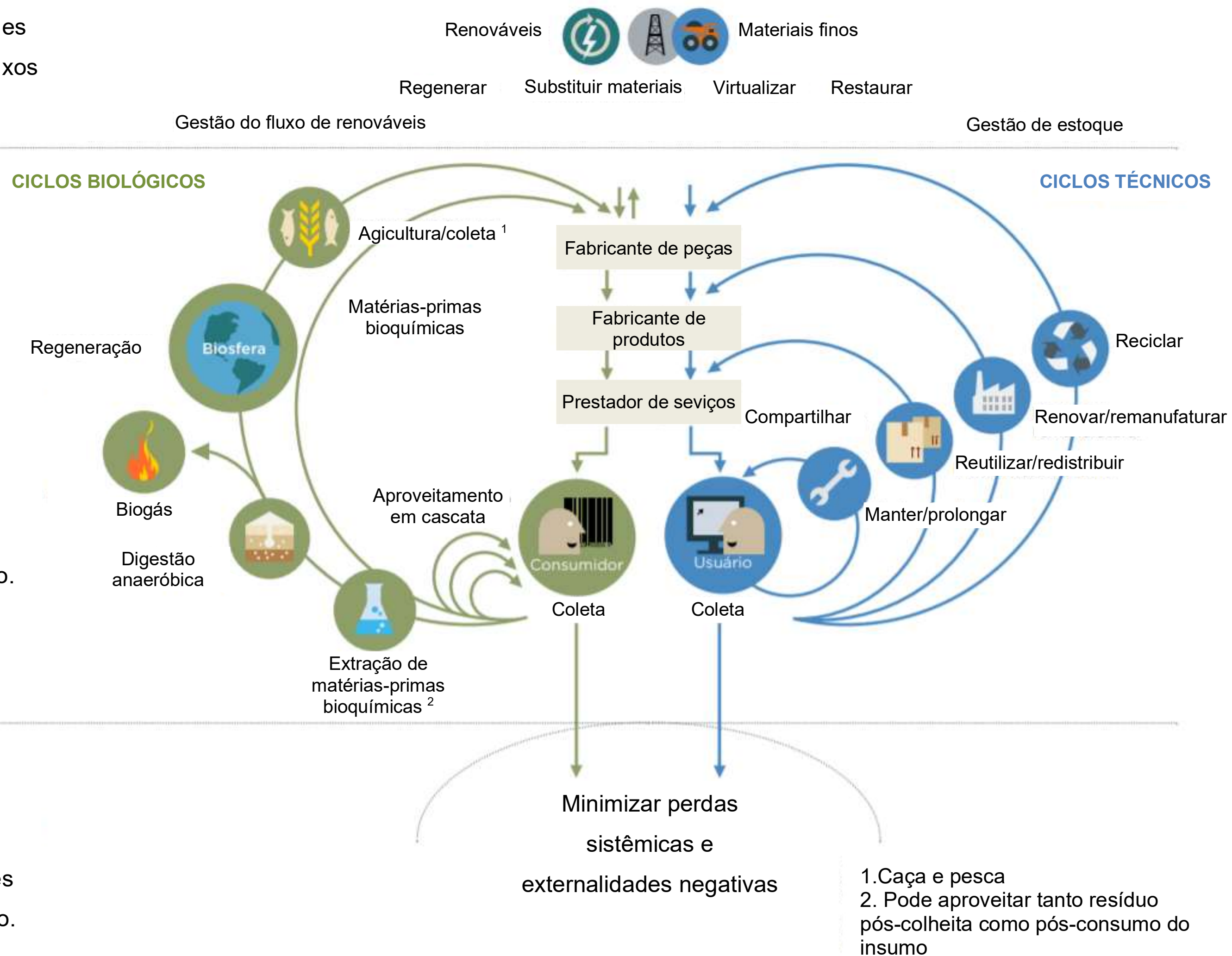


Figura 1. Modelo de economia circular. Fonte: Ellen Mac Arthur Foundation.

De acordo com FAO (2011), a perda de alimentos é definida como a redução da massa total de alimento comestível ao longo da cadeia de suprimentos (produção, pós-colheita, industrialização e distribuição), representando um desbaratamento de recursos utilizados nas etapas de produção (solo, água, energia, emissão desnecessária de CO_2) além da redução do valor econômico. Por sua vez desperdício, representa a perda de alimentos que ocorre no varejo ou no consumidor final (FAO, 2011). A diretriz da União Europeia para manejo de desperdício (2008) define co-produto como qualquer substância ou objeto resultante de processos produtivos cujo objetivo principal não é sua geração. É necessário ser passível de uso subsequente, legalmente permitido e poder ser trabalhado com processos industriais (UE, 2008). Nesse cenário, a redução, a gestão e a valorização de resíduos agroindustriais ganha destaque, como forma de reduzir o desperdício e a perda de alimentos que devem ser utilizados como co-produtos para recuperar compostos funcionais e desenvolver novos produtos de maior valor agregado (GALANAKIS, 2012). Os co-produtos da indústria de alimentos contêm componentes de elevado valor agregado, como proteínas, polissacarídeos, fibras, compostos aromáticos e fitoquímicos, que uma vez extraídos, podem ser reutilizados como ingredientes com função nutricionais e farmacológicas (ALEXANDRE et al., 2018).

2. Culturas Oleaginosas

No âmbito de grão oleaginosos, os de maior destaque são: soja, canola, girassol, amendoim, algodão e palmiste, segundo o relatório do Serviço para Agricultura Estrangeira (Foreign Agricultural Service, FAS) do Departamento de Agricultura dos Estados Unidos (USDA). A produção mundial de oleaginosas na safra de 2017/18 foi de 580,7 milhões de toneladas e no mercado internacional o Brasil assume uma posição de destaque, sendo o segundo maior produtor (125,8 milhões de toneladas) e o maior exportador (76,49 milhões de toneladas) (USDA, 2018). A Tabela 1 mostra os dados comparativos da produção mundial de oleaginosas, óleo vegetal e farelo.

Farinhas desengorduradas de oleaginosas contém alto teor de proteína e tem sido utilizadas para suplementar a dieta humana, sendo frequentemente aplicadas na fortificação de produtos de panificação a base de cereais (LAI et al., 2017). As proteínas vegetais são um insumo para a indústria de alimentos apresentando propriedades tecnológicas relevantes para formulações de produtos, tais como, capacidades emulsificante, modificadora de textura e de absorção de água e gordura (OGUNWOLU et al., 2009). O estudo e a aplicação das proteínas vegetais, bem como a produção de isolados

e concentrados proteicos vegetais está em ascendência devido ao público crescente nas vertentes veganas e vegetarianas além da preocupação com o volume de resíduos gerados após a extração de óleo das culturas oleaginosas (CHANG et al., 2014; KUSSMANN; PANCHAUD; AFFOLTER, 2010).

Tabela 1. Dados da safra mundial 2017/18 de sementes oleaginosas, óleo vegetal e farelo proteico.

	Produção Mundial (milhões de toneladas)		
	Sementes	**Óleo vegetal**	**Farelo**
Algodão	45,15	5,15	15,69
Palmiste	18,45	8,11	9,54
Amendoim	45,56	6,03	7,41
Canola	74,55	28,37	39,86
Soja	336,82	54,78	230,91
Girassol	47,46	18,39	19,76
Coco	-	3,66	-
Oliva	-	3,27	-
Palma	-	69,60	-
Total	580,70	198,78	331,53

Fonte: Adaptado de Relatório USDA, Julho de 2019.

Na categoria de nozes, conforme a Fundação Internacional para Nozes e Frutas Secas (International Nut and Dried Fruit Council Foundation - INC), foram produzidas 789.050 toneladas de castanha-de caju (sem casca) na safra de 2017/2018, sendo a terceira variedade mais produzida no mundo, atrás somente de amêndoas e nozes. O Estados Unidos é o maior produtos de nozes e castanhas, seguido por Turquia, China, Iran e Índia (INC, 2020). A Figura 2 mostra a produção das principais nozes.

Muitos estudos epidemiológicos e estudos clínicos mostram a correlação entre o consumo de diferentes tipos de nozes (amêndoa noz, avelã, noz pecã, pistache, macadâmia, castanha-de-caju e castanha do Pará) com a redução do risco de doenças crônicas não transmissíveis, como diabetes tipo 2 e doenças cardiovasculares (KIM; KEOGH; CLIFTON, 2017). Além disso, nozes e sementes comestíveis apresentam em sua composição ácidos graxos monoinsaturados (MUFA), tocoferóis, fitosteróis, carotenoides, compostos fenólicos e fibras, relacionados à propriedades antioxidantes, anti-inflamatórias e capazes de modelar a microbiota intestinal, podendo ter efeito benéfico para controle de obesidade e doenças inflamatórias (SUGIZAKI; NAVES, 2018).

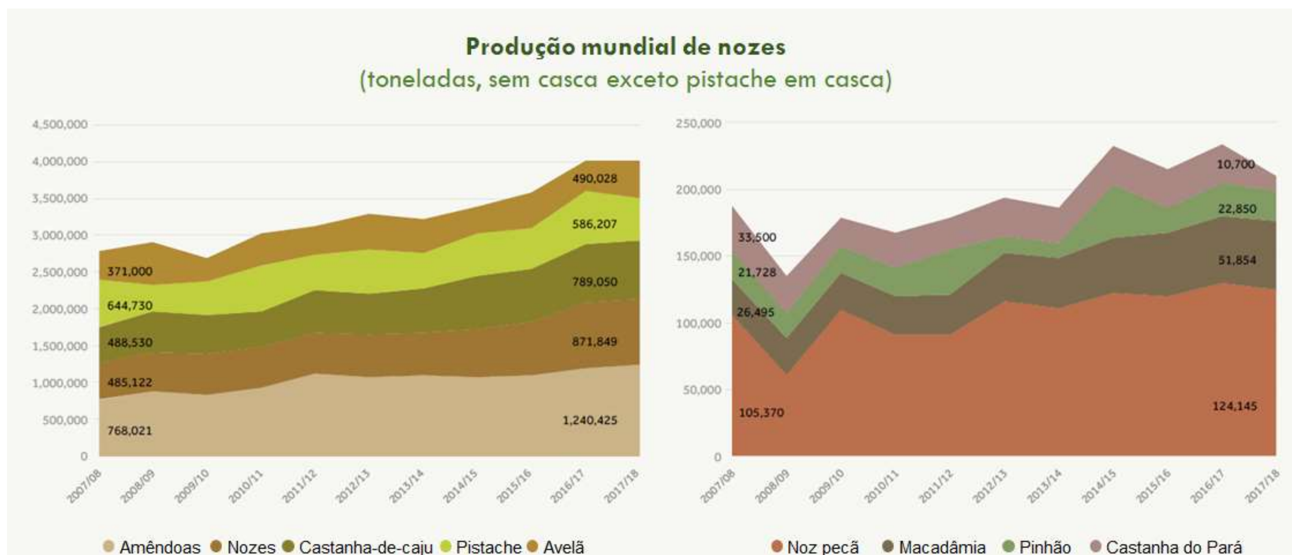


Figura 2. Gráfico da série histórica de produção de nozes e frutas secas. Fonte: adaptado de INC, 2020.

A presente pesquisa abordou o aproveitamento da torta desengordurada de dois grãos oleaginosos (girassol e palmiste) e de uma noz, a castanha-de-caju. Essas matrizes serão descritas em mais detalhes a seguir.

2.1. Girassol

O girassol (*Helianthus annuus L*.) é muito cultivado em função do alto rendimento em óleo comestível. A produção do óleo resulta numa torta residual com elevado conteúdo proteico (30-50% em matéria seca) (ALBE SLABI et al., 2019). No Brasil, o cultivo dessa oleaginosa se concentra nas regiões centro-oeste, sudeste e sul do país, como representado na Figura 3 (CONAB, 2018).

As proteínas da semente de girassol são em sua grande maioria globulinas (de 40% a 90%), seguida pelas albuminas (de 10% a 30%), sendo 11S globulina (heliantinina) e 2S albumina (albuminas do girassol) as frações majoritárias (PICKARDT et al., 2015). O teor de aminoácidos essenciais (exceto pela lisina) atende aos padrões definidos pela FAO, caracterizando o farelo de girassol uma fonte proteica de elevada qualidade nutricional (ALEXANDRINO et al., 2017).

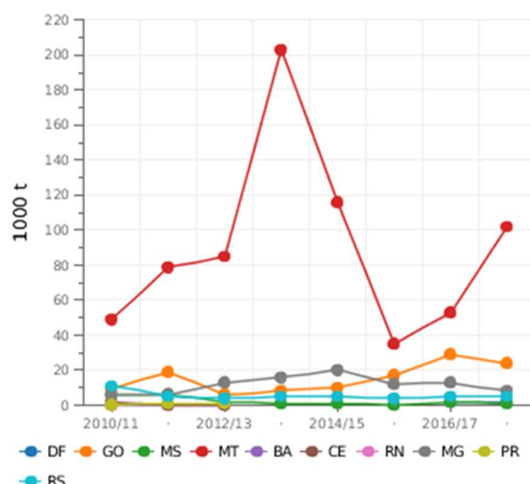


Figura 3. Demonstrativo da produção de girassol por estado brasileiro em milhões de toneladas. Fonte: Conab, 2018.

A torta de girassol, apresenta potencial de aproveitamento para a alimentação humana sendo uma dificuldade para tal, o elevado teor de compostos fenólicos (de 1 a 4g/100g). Dentre estes, existe a prevalência do ácido clorogênico, que confere ao insumo coloração verde escuro e características organolépticas indesejáveis (ALEXANDRINO et al., 2017; PICKARDT et al., 2015). O ácido clorogênico é formado por um grupo de ésteres de ácido hidroxicinamico, como os ácidos caféico, ferúlico e cumárico, com ácido quínico (BODOIRA; MAESTRI, 2020). A estrutura química desses compostos está demonstrada na Figura 4. A estrutura química do ácido clorogênico o torna efetivo para sequestro de radicais livres e atividade anti-mutagênica (PISOSCHI; POP, 2015).

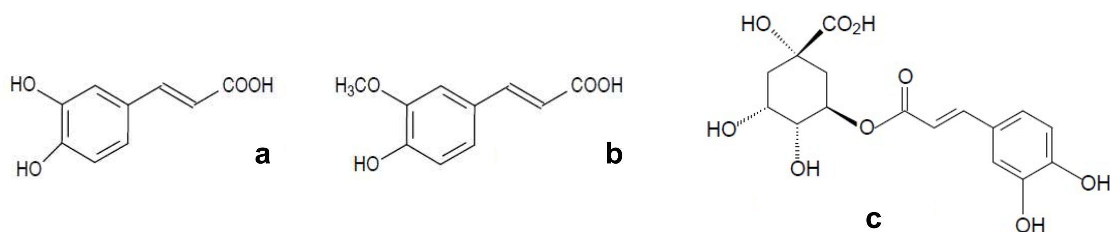


Figura 4. Estruturas de (a) ácido caféico, (b) ferúlico e (c) clorogênico. Adaptado de Bodeira & Maestri (2020).

2.2. Palmiste

A palmeira oleaginosa (*Elaeis guineensis Jacq.*) é uma das espécies mais cultivadas comercialmente para a produção de óleo de palma e de palmiste a partir do mesocarpo e do endosperma do fruto, respectivamente (SABIHA-HANIM; NOOR; ROSMA,

2011). A torta de palmiste ("palm kernel cake", PKC) é o co-produto agroindustrial majoritário da indústria de óleo de palma (KALIDAS et al., 2017), chegando ao montante de 9,77 milhões de toneladas no ano de 2017/18 (USDA, 2018). O descarte do PKC é um dos desafios para a indústria de óleo de palma e sua valorização é uma etapa importante para reduzir a poluição ambiental (BELLO et al., 2018). A Figura 5 ilustra produto e co-produto do cultivo de palmeira oleaginosa.

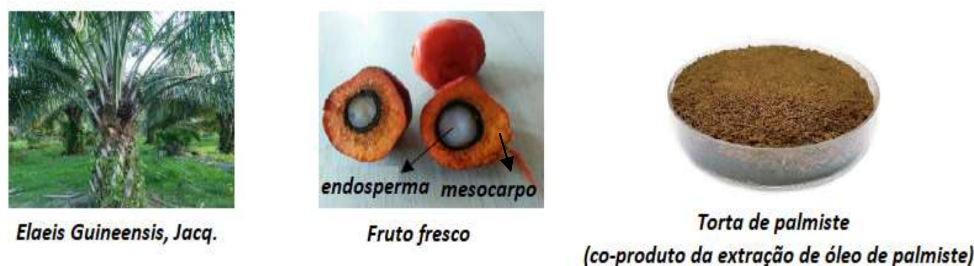


Figura 5. Ilustração da palmeira produtora de óleo de palma, a fruta fresca e o co-produto da extração de óleo de palmiste. Adaptado de Bello et al., 2018.

A semente *in natura* de palmiste contém baixo teor de açúcares simples (2.4%) e 81% de carboidratos não-amiláceos, com prevalência de mananas (78%) com baixo grau de substituição por resíduos de galactose, seguido de celulose (12%) e glucoronoxilanas e arabinoxyladas (3% cada) (ZHANG et al., 2018). A PKC apresenta de 14,6 - 22% de fibra total, composta principalmente por polissacarídeos não-amiláceos de manana, (1-4)-β-d-manopiranosil, com baixa substituição por galactose (12-20%), elevada cristalinidade e baixa solubilidade em água. É comercialmente utilizada para recuperação de celulose, hemicelulose e lignina ou para alimentação de ruminantes, suínos e piscicultura (CERVERÓ et al., 2010; KALIDAS et al., 2017). A fibra extraída de torta desengordurada de palmiste tem sido utilizada para produzir pães e cookies com alto teor de fibras (LAI et al., 2017).

Estudo do efeito proliferativo de bactérias ácido láticas por polissacarídeos extraídos do PKC (*Lactobacillus plantarum* ATCC8014 e *Lactobacillus rhamnosus* ATCC 53103) observaram crescimento das bactérias e acidificação do meio de cultura, com a produção de ácidos graxos de cadeia curta, necessitando maior investigação para compreender o mecanismos de ação pelo qual as bactérias utilizam o substrato (BELLO et al., 2018). Outro estudo relatou viabilidade de extrair manano-oligosacarídeos (MOS) de PKC com efeito proliferativo de *Lactobacillus reuteri* C1 (KALIDAS et al., 2017).

Por seu baixo valor econômico, PKC pode ser valorizado como matéria-prima para extração de proteína e peptídeos de origem vegetal (LING et al., 2013). A torta de palmiste

apresenta de 16% a 18% de proteína residual, podendo constituir uma fonte de peptídeos antibacteriano (TAN; AYOB; WAN YAACOB, 2013). Tais peptídeos são caracterizados como peptídeos catiônicos, ou seja, que contém teor de aminoácidos positivamente carregados (como arginina, histidina e lisina) capazes de interagir com os fosfolipídios negativamente carregados da parece celular de bactérias, inserindo-se no seu citoplasma e exercendo assim sua atividade (TAN; AYOB; WAN YAACOB, 2013). O estudo de Chang et al. (2014) demonstrou através da técnica de eletroforese em gel, uma predominância de glutelinas nas proteínas totais do palmiste. Estudo isolou dois peptídeos a partir da PKC (sequências aminoacídica GIFE e LPWRPATNVF) que sintetizados apresentaram atividade antioxidante por DPPH e quelante de metais (ZAREI et al., 2014).

2.3. Castanha de Caju

O cajueiro (*Anacardium occidentale L.*) é uma planta nativa do nordeste brasileiro muito cultivada atualmente na África e Índia Ocidental, adaptando-se bem a solos de baixa fertilidade, ambientes com temperaturas elevadas e estresse hídrico (OGUNGBENLE; AFOLAYAN, 2015). Constitui-se como uma importante fonte de renda para os estados do nordeste brasileiro, sendo o principal produto, a amêndoa localizada no interior da castanha-de-caju (fruto) (SERRANO & PESSOA, 2016). A película que reveste a amêndoa destina-se a indústria química de tintas, vernizes e lubrificantes e o pedúnculo do caju (pseudofruto) é processado por indústrias de bebidas e polpas de frutas (SERRANO & PESSOA, 2016). A Figura 6 ilustra as subdivisões do fruto e pseudofruto. Por compor a biodiversidade brasileira, o estudo com *Anacardium occidentale* e seus derivados foi cadastrado no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento tradicional Associado (SisGen).

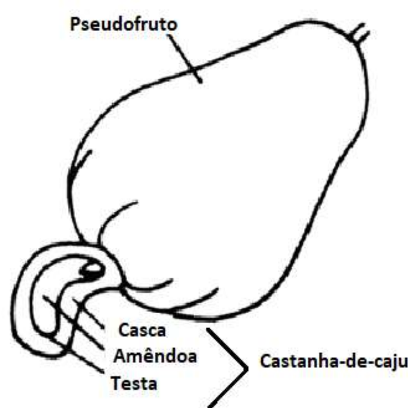


Figura 6. Subdivisões do fruto e pseudofruto do cajueiro. Adaptado de Embrapa (2006).

Nozes da família *Anacardiaceae* (pistache e castanha-de-caju) têm sido consumidas em função de aspectos organolépticos e de benefício a saúde, sendo incluídas em uma vasta gama de produtos, como produtos de panificação, sorvete, molhos, doces e iogurtes. As farinhas são utilizada como substituto de trigo em formulações de produtos sem adição de glúten (SANCHIZ et al., 2019). Estudos epidemiológicos mostram que o consumo de nozes reduz o colesterol sérum total e o LDL. Esse efeito pode ser atribuído não exclusivamente à composição dos ácidos graxos, mas também a proteínas (com destaque ao aminoácido arginina), fibras, fitosteróis, antioxidantes, folatos e minerais (cobre, magnésio, potássio e boro) (KAMAL-ELDIN; MOREAU, 2009).

O beneficiamento da castanha-de-caju pode gerar até 40% de castanhas quebradas, que tem menor valor de mercado que as castanhas inteiras e, devido ao elevado valor nutricional, podem ser utilizadas como ingredientes na formulação de outros produtos alimentícios (DE CARVALHO et al., 2018) ou ainda para produção de óleos comestíveis de elevado valor agregado (EMBRAPA, 2018). Os óleos de nozes são apreciados em função de suas características sensoriais, sendo mais caros que demais óleos gourmets. No processo de extração desses óleos utiliza-se a prensagem a frio, solventes ou extração por fluído super crítico (EFS), utilizando-se geralmente o gás carbônico e algumas vezes modificado por adição de etanol. (KAMAL-ELDIN; MOREAU, 2009). Dentre os solventes utilizados em processos industriais para extração de óleo destaca-se o n-hexano. Porém o etanol apresenta vantagens como substituto desse solvente, principalmente por não ser tóxico e não ser derivado do petróleo (EMBRAPA, 2018).

O óleo extraído por prensa a frio (PF) apresenta melhor características organolépticas, sendo denominado como "óleo extra virgem de noz". Solventes são utilizados para extrair óleo a partir de nozes de baixa qualidade ou da biomassa pós prensa a frio, obtendo-se óleos qualidade organoléptica inferior aos obtidos por PF. Após o uso de solvente são necessárias etapas de refino, como neutralização, branqueamento, degomagem e desodorização. A EFS produz óleos geralmente mais claros, com maior teor de tocoferol e ligeiramente mais estáveis, comparados aos obtidos por aplicação de solvente, que apresenta um rendimento um pouco superior. A extração por solvente ou EFS não apresentam diferença quanto a composição de lipídeos e esteróis. (KAMAL-ELDIN; MOREAU, 2009). O óleo de castanha-de-caju apresenta em sua maioria ácidos graxos insaturados, sendo os ácidos graxos majoritários os ácidos oléico (C18:1), linoléico (C18:2), palmítico (C16:0) e esteárico (C18:0) (EMBRAPA, 2018).

Segundo estudo de Ogungbenle e Afolayan (2015), as amêndoas da castanha-de-caju contém cerca de 43% de óleo e 26% de proteína e a farinha de castanha-de-caju (após extração do óleo) apresentam alto teor de proteína (45%) e de aminoácidos essenciais como lisina e metionina, baixo teor de fibras (2%) e aproximadamente 6% de minerais. Compostos bioativos das amêndoas de castanha-de-caju, como fenólicos, flavonoides, fosfolipídios, esteróis e tocoferóis, demonstração ação antioxidante (CHANDRASEKARA; SHAHIDI, 2011).

3. Compostos bioativos e bioatividades

O avanço da ciência possibilitou compreender o impacto da dieta na regulação do metabolismo humano e suas consequências na expressão gênica e molecular, o que ocasionou uma revolução nas estratégias alimentares e na ciência nutricional, com a criação de novos conceitos, como o de compostos bioativos e nutrição funcional (BIESALSKI et al., 2009). Em vegetais, define-se duas categorias de metabólitos principais. Metabólitos primários são substâncias químicas com funções principais de promover o crescimento e desenvolvimento (como carboidratos, proteínas e lipídeos) e metabólitos secundários são compostos que permitem à planta maior adaptação e sobrevivência ao ambiente, frequentemente exercendo efeitos em sistemas biológicos, sendo considerados compostos bioativos (AZMIR et al., 2013). Tais compostos estão presentes em pequenas concentrações principalmente em frutas, vegetais, cereais e possuem potencial terapêutico com modulação de processos metabólicos, tais como a redução de estado pró-inflamatório, estresse oxidativo e desordens metabólicas (ROSELLÓ-SOTO et al., 2016).

Uma nutrição equilibrada e o acesso adequado a alimentos de elevada qualidade nutricional são fundamentais para o crescimento e desenvolvimento de crianças, para a manutenção das funções do corpo humano além contribuir para a prevenção de doenças crônicas não-transmissíveis (DCNT) (FAO,2013). O desbalanço nutricional e condições ambientais desfavoráveis podem, por meio de rotas metabólicas, promover a alterações na expressão gênica que, a longo prazo, aumentam o risco de desencadear fisiopatologias, doenças e síndromes que representam um grande problema de saúde pública no mundo, sendo as mais frequentes: obesidade, diabetes, hipertensão, doenças cardiovasculares, asma e alergias, além de doenças imune e autoimunes, doenças que acometem o desenvolvimento neurológico, doenças neurodegenerativas e alguns tipos de câncer (BAROUKI et al., 2012). Estudos epidemiológicos mostram que o consumo de alimentos ricos em compostos bioativos com atividade antioxidante (incluindo vitaminas e principalmente compostos fenólicos) tem um efeito positivo na saúde humana podendo

reduzir o risco de cânceres, doenças cardiovasculares, Alzheimer, diabetes, catarata e disfunções correlacionadas com o envelhecimento (ROSELLÓ-SOTO et al., 2016).

A demanda por alimentos que contribuam para um estilo de vida saudável impulsionou a indústria a desenvolver alimentos funcionais, por exemplo, pela reformulação de alimentos com redução de gorduras trans, menor teor de sal e açúcar e incorporação de ingredientes funcionais, como probióticos e prebióticos (SOUSA et al., 2015; TURGEON; RIOUX, 2011). Contudo, o desenvolvimento de alimentos funcionais deveria ser intimamente associado a conceitos de biodisponibilidade e bioacessibilidade dos nutrientes, condições prévias para que exerçam sua bioatividade (CILLA et al., 2018). Segundo Fernández-García e colaboradores (2009), a biodisponibilidade é a porção da quantidade total de alimento ingerida que pode ser assimilada pelo corpo, para funções metabólicas e/ou ser armazenada, englobando os conceitos de bioacessibilidade e bioatividades. A bioacessibilidade inclui todas as etapas de digestão gastrointestinal nas quais ocorre a transformação do alimento em um material capaz de ser absorvido pelas células do epitélio intestinal, e sua pré-metabolização no intestino e fígado (FERNÁNDEZ-GARCÍA; CARVAJAL-LÉRIDA; PÉREZ-GÁLVEZ, 2009). No entanto, a bioatividade depende de como os possíveis compostos bioativos são transportados e atingem os tecidos de interesse, interagindo com biomoléculas e que, quando metabolizados, geram biomarcadores os quais são capazes de modular e amplificar respostas fisiológicas, produzindo benefícios à saúde (FERNÁNDEZ-GARCÍA; CARVAJAL-LÉRIDA; PÉREZ-GÁLVEZ, 2009).

Em 2014, um consenso internacional de pesquisadores científicos propôs um protocolo para padronização da digestão humana simulada *in vitro* (MINEKUS et al., 2014) posteriormente atualizado (BRODKORB et al., 2019). A biodisponibilidade por digestão *in vitro* é primordial para avaliar estabilidade, digestibilidade, mudanças estruturais, efeitos de matriz e de processamento dos alimentos sobre os compostos alvos de estudo e elucidar as vias metabólicas de atuação (metabolismo) e absorção, através de ensaios com culturas celulares ou fermentação do cólon *in vitro* (MOTILVA; SERRA; RUBIÓ, 2015). Sabe-se, contudo, que a biodisponibilidade não depende apenas do processo mecânico e enzimático do trato gastrointestinal, podendo ser influenciada pela composição da microbiota intestinal (LOGAN; KATZMAN, 2005).

3.1. Probióticos e Prebióticos

Os alimentos a serem digeridos no intestino ficam separados das células epiteliais do cólon por uma final camada de muco (JONES; NEISH, 2017). As células epiteliais se

renovam constantemente e para captarem os nutrientes dos alimentos estão frequentemente em contato com o conteúdo do lúmen, sendo danificadas por exposição a patógenos e eventos imunologicamente danosos (JONES; NEISH, 2017). A imunidade inata prevê a homeostase das células epiteliais com um balanço estreito de resposta e tolerância a microrganismos no lúmen intestinal (SANSONETTI, 2004).

Em geral, o estômago apresenta poucas unidades formadoras de colônias bacterianas por mililitros de suco gástrico e ocorre um aumento da concentração de bactérias ao decorrer do intestino, atingindo a contagem de 10^{12} UFC/g no intestino grosso (cólon) (FAO, 2006). O intestino humano é colonizado por um complexo conjunto de bactérias, chamada microbiota residente (GIBSON, 2004). A microbiota coloniza principalmente o cólon em uma relação de simbiose, realizando fermentação sacarolítica e proteolítica de componentes dos alimentos não digeridos no trato gastrointestinal superior (como amido resistente, carboidratos não digeríveis, oligossacarídeos, proteínas e mucilagens) utilizando tais substratos como fonte de energia para seu metabolismo e liberando metabólitos que podem afetar positiva ou negativamente à saúde humana (BLAUT; CLAVEL, 2007; GIBSON, 2004; KAU et al., 2011).

Determinas espécies colonizadoras, eventualmente patógenas, podem ter correlação com produção de compostos carcinogênicos, formação de compostos tóxicos com efeitos de constipação ou diarreico, além de dano ao fígado (MANNING; GIBSON, 2004), desordens metabólicas e inflamatórias crônicas, incluindo a síndrome do intestino irritado (CLAESSON et al., 2012) e síndrome metabólica, incluindo obesidade e diabetes mellitus tipo II (QIAO et al., 2013). A anomalia de quantidade e diversidade de bactérias no microbioma (disbiose) pode ser suficiente para agravar doenças inflamatórias intestinais, doenças infecciosas (colites pseudomembranosas), desordens imunológicas sistêmicas, doenças alérgicas (doença celíaca e asma) e doenças metabólicas em adultos (diabete e obesidade) (BHATTACHARYYA et al., 2014). Contudo, *Bifidobacterium sp.* e *Lactobacillus sp.* são exemplos de gêneros da microbiota que podem influenciar favoravelmente a saúde do hospedeiro, por diferentes mecanismos, podendo ser destacados a produção de ácidos graxos de cadeia curta (AGCC), melhora da digestão e absorção de energia residual de componentes ingeridos na dieta, síntese de vitaminas, exclusão competitiva de microrganismos patógenos, redução do nível de colesterol sérico, redução da formação de gases além de correlacionar-se a respostas do sistema imune e influenciar a homeostase do tecido ósseo (MANNING; GIBSON, 2004; JONES & NEISH, 2017).

Fazer uso da dieta para controlar desordens de saúde e modular a microbiota intestinal é uma abordagem comum em ciências nutricionais além de atrair consumidores

(VIEIRA DA SILVA; BARREIRA; OLIVEIRA, 2016). Assim, uma abordagem mercadológica bastante difundida é veicular probióticos (bactérias majoritariamente dos gêneros *Bifidobacterium sp.* e *Lactobacillus sp.*) e prebióticos em alimentos (CLAESSON et al., 2012).

Segundo a FAO/WHO (2006), probióticos são micro-organismos vivos, que quando administrados em quantidades adequadas conferem benefícios à saúde do hospedeiro. De acordo com o consenso da Associação Cinentífica Internacional em Probióticos e Prebióticos (ISAPP), prebióticos são "substratos que utilizados seletivamente por microorganismos do hospedeiro conferem benefícios à saúde" e efeito prebiótico é "o estímulo seletivo de bifidobacteria, lactobacillus ou outras espécies além desses gêneros que evoquem um benefício mensurável à saúde do hospedeiro, em relação a um controle" (GIBSON et al., 2017). Um grupo de pesquisadores revisou a taxonomia de bactérias pertencentes às famílias *Lactobacillaceae* e *Leuconostocaceae*, conforme o sequenciamento do genoma. Dessa forma, bactérias que pertenciam ao gênero *Lactobacillus* foram redistribuidos nos gêneros *Lactobacillus delbrueckii* e *Paralactobacillus*, além de em novos 23 gêneros chamados *Holzapfelia, Amylolactobacillus, Bombilactobacillus, Companilactobacillus, Lapidilactobacillus, Agrilactobacillus, Schleiferilactobacillus, Loigolactobacilus, Lacticaseibacillus, Latilactobacillus, Dellaglioa, Liquorilactobacillus, Ligilactobacillus, Lactiplantibacillus, Furfurilactobacillus, Paucilactobacillus, Limosilactobacillus, Fructilactobacillus, Acetilactobacillus, Apilactobacillus, Levilactobacillus, Secundilactobacillus* e *Lentilactobacillus* (ZHENG et al., 2020).

Dentre os substratos prebióticos e possíveis candidatos a tal estão a lactulose, fruto-oligosacarídeos (FOS), galacto-oligosacarídeos (GOS), mananoligosacarídeos (MOS), xilo-oligosacarídeos (XOS), oligossacarídeos da soja (SOS), gluco-oligosacarídeos, isomalto-oligosacarídeos (IMO), oligossacarídeos do leite humano (HMO), pectina, ácido linolênico conjugado (CLA), ácido graxos poli-insaturados (PUFA), amido resistente, álcoois, compostos fenólicos, fitoquímicos e fibras dietéticas (GIBSON et al., 2017). Os carboidratos dietéticos (açúcares, amido e polissacarídeos não-amiláceos) podem ser classificados como digeríveis e não-digeríveis, podendo sua digestibilidade ser limitada por estruturas vegetais específicas que dificultam o acesso das enzimas gastrointestinais (LEONG et al., 2019). Nesse sentido técnicas de processamento adequadas, como moagem, podem melhorar a digestibilidade (LEONG et al., 2019). A Figura 7 resume o tipo de carboidratos presentes na dieta.

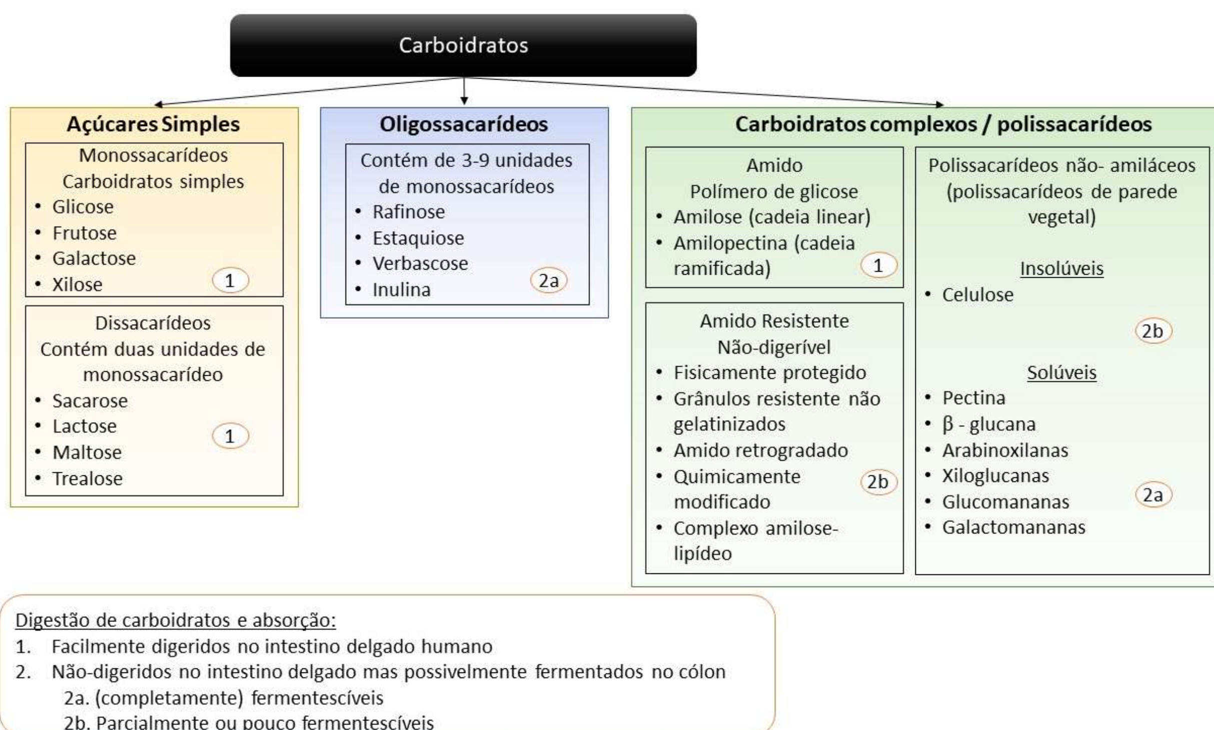


Figura 7. Carboidratos dietéticos e o potencial de digestão e absorção no corpo humano. Adaptado de Leong et al. (2019).

No trato gastrointestinal, o amido é rapidamente digerido no intestino delgado porém, amido resistente (AR) e polissacarídeos não-amiláceos (PSNA; polímeros constituintes da parede celular vegetal e formados por diferentes monossacarídeos ligados por ligações β-glicosídicas) não sofrem digestão pelas enzimas humanas (LOVEGROVE et al., 2017). Dessa forma, AR e PSNA são os principais componentes das fibras dietéticas, capazes de atingirem o cólon e serem fermentadas pela microbiota, produzindo ácidos graxos de cadeia curta, como butirato, propionato e acetato (LOVEGROVE et al., 2017). A composição monomérica, o grau de polimerização, o tipo de ligação glicosídica, bem como o peso molecular dos polissacarídeos e oligossacarídeos podem influenciar o efeito de proliferação dos probióticos (BELLO et al., 2018). Napolitano e colaboradores (2006) demostraram que a conversão de fibras dietéticas insolúveis em fibras solúveis por meio de prévia hidrólise enzimática, libera composto fenólico antes ligados a polissacarídeos e ligninas, com o efeito de aumentar a biodisponibilidade de fenólicos totais solúvel em água e aumento da atividade antioxidante por método DPPH. Saura-Calixto (2011) propôs que fibras e antioxidantes deveriam ser abordados em conjunto, uma vez que aproximadamente 50% dos compostos fenólicos são absorvidos no intestino delgado estando ligados a fibras.

O efeito prebiótico *in vitro* pode ser determinado medindo-se o estimulo à proliferação de bactérias probióticas pelo substrato que se deseja investigar. O meio de

cultura Man-Rogosa-Sharpe (MRS) foi desenvolvido com o propósito de ser um meio seletivo para bactérias ácido-láticas (“lactic acid bactéria”, LAB) capaz de oferecer melhores condições de crescimento principalmente de lactobacilli (comparado por exemplo ao meio de cultura Briggs, utilizado anteriormente) (DE MAN; ROGOSA; SHARPE, 1960). Pode ser feito o inóculo em meio de cultura MRS e após o período de incubação, conta-se o número de unidades formadoras de colônias (UFC), a densidade dos micro-organismos expressa em UFC/g e o potencial efeito prebiótico comparando-se com um controle, por meio de análise estatística (HUANG et al., 2012).

3.2. Capacidade antioxidante

Segundo a literatura, “antioxidantes são substâncias que, quando presentes em mais baixa concentração comparada a da espécie oxidante no meio, inibem a oxidação do substrato” (GRANATO et al., 2018). São compostos extremamente importantes na indústria de alimentos, onde são empregados para retardar a peroxidação lipídica e a produção de compostos secundários que podem influenciar negativamente o sabor, a textura e a cor do produto, contribuindo para estender o tempo de vida de prateleira, na indústria de cosméticos e fármacos, bem como para o organismo humano (SAMARANAYAKA; LI-CHAN, 2011). Pró-oxidantes, como espécies reativas de oxigênio (ERO) e de nitrogênio (ERN), ocorrem naturalmente em células aeróbicas em função do seu metabolismo aeróbico normal e por isso, possuem o próprio sistema de defesa antioxidante (enzimático e não enzimático) para manutenção da homeostase redox (SHEIH; WU; FANG, 2009).

Fontes endógenas de ERO são formadas durante a redução tetravalente do oxigênio molecular em água dentro das mitocôndrias ($O_2^{-\bullet}$, $HO_2^{\bullet}$, OH e H_2O_2), por enzimas catalizadoras de ERO como a peroxidase, NADPH oxidase, NADPH oxidase isoformas (NOX), xantina oxidase (XO), lipoxigenases (LOXs), glucose oxidase, mieloperoxidase (MPO), óxido nítrico sintetase e ciclo-oxigenases (COXs) (BHATTACHARYYA et al., 2014). As ERO podem ser espécies radicalares (as quais possuem um ou mais elétrons desemparelhados na camada de valência, como o ânion superóxido ($O_2^{-\bullet}$), radical hidroxila (OH), radical alcaloxil ($RO\cdot$) e radical peroxil ($ROO\cdot$)) ou não-radicalares (como peróxido de hidrogênio (H_2O_2) e oxigênio singlete ($^1O_2^-$)) (LEE; KOO; MIN, 2004). As espécies reativas de nitrogênio (ERN) contemplam, por sua vez, o óxido nítrico ($NO\cdot$), dióxido de nítrico ($NO_2\cdot$) e peróxido nitrito ($OONO^-$) (LEE; KOO; MIN, 2004). Poluentes presentes no ar e em cigarros, bem como a radiação UV podem estimular a geração de radicais livres na pele, capazes de ser absorvidos e assim acessarem a corrente sanguínea (SARMADI; ISMAIL, 2010).

O sistema antioxidante endógeno pode ser classificado em enzimático (composto pelas enzimas superóxido dismutase, catalase, glutationa peroxidase, glutationa redutase, peroxiredoxinas) e não-enzimático intracelular (incluindo compostos como ferritina, mioglobina, metalotioneínas, glutationa, coenzima Q_{10}, melatonina, poliaminas) e extracelular (transferrina, lactoferrina, albumina, seruloplasmina e ácido úrico) (MIROŃCZUK-CHODAKOWSKA; WITKOWSKA; ZUJKO, 2018; PISOSCHI; POP, 2015). O desbalanço da homeostase redox a favor do sistema pró-oxidante caracteriza o estado de estresse oxidativo, ocasionando danos a macromoléculas de proteína, lipídeos e do DNA, culminando em mutações genéticas e no desenvolvimento de doenças crônicas não-transmissíveis (DCNT) (ZHANG; MU; SUN, 2014). Nesse cenário nota-se a relevância dos compostos antioxidantes exógenos.

Antioxidantes dietéticos como vitamina E, vitamina C, carotenoides, alguns minerais (Zn, Mn, Cu, Se) e polifenóis (flavonoides, ácidos fenólicos, estibenos e ligninas) encontrados em fruitas, vegetais, bebidas (chás, café), nozes e cereais podem desenvolver ação sinérgica com o sistema antioxidante endógeno para manter e/ou reestabelecer a homeostase redox, contribuindo assim para prevenção e/ou postergação do aparecimento de DCNT, como diabetes tipo II, Mal de Alzheimer, Parkinson, desordens cardiovasculares, envelhecimento e alguns tipos de câncer (CANABADY-ROCHELLE et al., 2015; GRANATO et al., 2018; MIROŃCZUK-CHODAKOWSKA; WITKOWSKA; ZUJKO, 2018; VIEIRA DA SILVA; BARREIRA; OLIVEIRA, 2016). Peptídeos e fibras dietéticas também tem demonstrado atividade antioxidante (ACOSTA-ESTRADA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2014; ALASALVAR; BOLLING, 2015; MRABET et al., 2017; SARMADI; ISMAIL, 2010).

Alguns ensaios químicos *in vitro* tem o propósito de investigar, de forma rápida, simples, eficaz e com menor custo, as possíveis atividade antioxidantes de compostos alimentares (GRANATO et al., 2018). Para essa finalidade são utilizados inúmeros métodos e dentre os mais difundidos estão o de sequestro do radical 2,2 difenil-1-pricril-hidrazil ($DPPH^{\bullet}$), o de sequestro do radical 2,2-azino-bis (3-etilbenzotiazolin)-6-sulfônico ($ABTS^{\bullet+}$), da capacidade de absorção de radicais oxigênio (ORAC), e ensaio de proteção ao DNA (GRANATO et al., 2018). A determinação da capacidade antioxidante de amostras biológicas depende do tipo de radical livre ou oxidante utilizado (AL-DUAIS et al., 2009). Uma desvantagem dos ensaios *in vitro* para determinação de atividade antioxidante é a falta de mimetização das condições fisiológicas do corpo humano e por não considerar a bioacessibilidade dos compostos antioxidantes (WOLFE; RUI, 2007).

O ensaio DPPH baseia-se na redução do radical DPPH• pela ação da substância antioxidante a ser testada (doadora de um átomo de hidrogênio ou um elétron), ocorrendo a mudança de coloração (de violeta escuro a amarelo claro) com alteração da absorbância a 515 nm (AL-DUAIS et al., 2009; W. BRAND-WILLIAMS, 1995). O ensaio de ABTS baseia-se na geração do radical 2,2-azino-bis (3-etilbenzotiazolin)-6-sulfônico (de cor verde) pela reação de ABTS com perssulfato de potássio para então reagi-lo com o antioxidante (em pH próximo da neutralidade), que ocasionará a perda de coloração e redução da absorbância a 730 nm (AL-DUAIS et al., 2009).

Diferentemente dos ensaios colorimétricos abordados anteriormente, o ensaio de ORAC baseia-se na capacidade de redução do radical peroxil (ROO•) gerado pela degradação térmica do 2,2'-azobis(2-amidino-propano) di-hidroclorado (AAPH) e na preservação da molécula de fluoresceína pelo antioxidante frente a ação do radical peroxil, monitorando-se o decaimento de fluorescência em pH 7,4 (CHISTÉ et al., 2011). Preconiza ser um ensaio mais relevante por utilizar o radical mais comum em sistemas biológicos (AL-DUAIS et al., 2009).

Biomarcadores de peroxidação lipídica e proteica, bem como ensaio de dano ao DNA, podem ajudar a monitorar os danos do estresse oxidativo *in vivo* (SARMADI; ISMAIL, 2010). As lesões causadas por ERO à molécula de DNA variam desde a modificação às bases nitrogenadas até a indução à quebra das cadeias de DNA (SILVA et al., 2017). Nesse tipo de ensaio, a molécula de DNA de plasmídeo é inserida em meio reacional contendo o agente oxidante e composto antioxidante, reagindo à temperatura corpórea, para que por meio de eletroforese em gel de agarose 0,8% possam ser observadas as bandas de DNA "super-coiled" (estrutura original) e o aumento da intensidade na luz UV, das bandas circular e linear, formadas em função da quebra do DNA original (ZHANG; MU; SUN, 2014).

3.3. Peptídeos bioativos

O consumo de proteínas é fundamental para o desenvolvimento e manutenção da vida, equilibrando o balanço de nitrogênio e fornecendo aminoácidos necessários a diversas rotas metabólicas (WHO/FAO/UNU, 2007). O valor biológico das proteínas foi definido em função da composição aminoacídica e sua essencialidade, sendo relevante a presença ou não de fatores antinutricionais na matriz alimentar que reduzam a digestibilidade e absorção proteica (KITTS; WEILER, 2003; SARMADI; ISMAIL, 2010). Contudo, este conceito foi ampliado para considerar além do aspecto nutricional, a funcionalidade biológica, traduzida como a capacidade de gerar peptídeos a partir da

hidrólise da proteína original exibindo potencial de modular algumas funções fisiológica, como anti-inflamatória e anti-hipertensiva (BANERJEE et al., 2017). Estes fragmentos de proteína devem ser capazes de resistir à digestão gastrointestinal, chegando ao local de ação com estrutura intacta e em concentração suficiente para promover um efeito benéfico in vivo (HAYES, 2018).

Proteínas de origem animal e vegetal são fontes potenciais de uma vasta quantidade de peptídeos bioativos, presentes em suas estruturas (SÁNCHEZ; VÁZQUEZ, 2017). A partir das proteínas, os peptídeos bioativos podem ser gerados durante o processamento industrial, por hidrólises enzimáticas ou processos de fermentação. Tais peptídeos contém de 2 a 20 aminoácidos, massa molecular inferior a 6 kDa e sua bioatividade depende da composição aminoacídica da proteína original bem como de sua digestibilidade (resistência a enzimas digestivas), sendo que foram descritos na literatura peptídeos opióide, quelantes de metais, antioxidantes, antimicrobianos, moduladores do sistema imune, anti-hipertensivos, antitrombóticos e hipocolesterolêmicos, antidiabéticos, podendo um mesmo peptídeo desenvolver múltiplas funções (SÁNCHES; VAZQUÉZ, 2017TURGEON; RIOUX, 2011;). Ensaios *in sílico* tem sido utilizado para prever o potencial de bioatividade (NONGONIERMA; FITZGERALD, 2014).

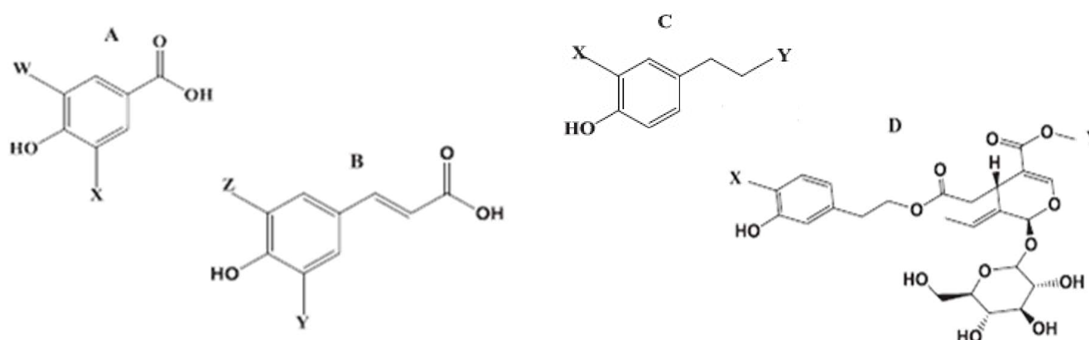
Peptídeos com atividade antioxidante apresentam baixo peso molecular, prevalência de aminoácidos hidrofóbicos (Alanina, Valina, Metionina, Fenilalanina, Leucina, Isoleucina, Prolina, Triptofano) em relação aos hidrofílicos (Arginina, Ácido Aspártico, Histidina, Lisina, Ácido Glutâmico) o que é considerado fundamental para o mecanismo antioxidante de sequestro de radicais (ZOU et al., 2016). Aminoácidos frequentemente relatados de desempenhar atividade antioxidantes são histidina, triptofano, fenilalanina, prolina, isoleucina, valina, metionina, tirosina (SÁNCHEZ; VÁZQUEZ, 2017; ZOU et al., 2016). Estudos demonstram que peptídeos de cadeia curta apesentam atividade antioxidante mais alta que frações de maior peso molecular (SÁNCHEZ; VÁZQUEZ, 2017).

3.4. Compostos fenólicos

Compostos fenólicos são metabólitos secundários de plantas, antioxidantes naturais e com atividade quelante, tendo em sua estrutura química característica anél(is) aromático(s) com variada substituição de grupos hidroxil (OH), ocorrendo de forma livre mas principalmente na forma ligada de ésteres ou glicosídica (VIEIRA DA SILVA; BARREIRA; OLIVEIRA, 2016). Podem ser divididos em cinco categorias principais: ácidos fenólicos, flavonoides, taninos, lignanas e estibenos (BODOIRA; MAESTRI, 2020), que são

facilmente solubilizados em água, etanol, metanol, acetona e outros solventes e capazes de retardar ou inibir a oxidação de DNA, proteínas e lipídeos (ALEXANDRE et al., 2018; VIEIRA DA SILVA; BARREIRA; OLIVEIRA, 2016).

Ácidos fenólicos simples tem habilidade para associar-se fortemente com metabólitos primários, como proteínas e carboidratos (ALU'DATT et al., 2017). O consumo de alimentos com alto teor de polifenóis tem sido associado com benefícios à saúde estando relacionados a propriedades anti-câncer, anti-microbiana e anti-inflamatória (SANCHIZ et al., 2019). A Figura 8 mostra a estrutura química dos principais ácidos fenólicos encontrados em culturas de oleaginosas.



A	W	X
Protocatequínico	H	OH
Vanílico	OCH_3	H
Seringico	OCH_3	OCH_3
Gálico	OH	OH
ρ - Hidroxibenzóico	H	H
B	**Y**	**Z**
ρ - Cumárico	H	H
Cafeico	H	OH
Ferúlico	H	OCH_3
Sinápico	OCH_3	OCH_3
C	**X**	**Y**
Tirosol	H	OH
Hidroxitirosol	OH	OH
Verbascoside	OH	Rhamnose
D	**X**	**Y**
Oleuropeína	OH	CH_3
Demetiloleuropeína	OH	H
Ligestroside	H	CH_3

Figura 8. Estrutura dos ácidos fenólicos (A, B, C e D) encontrados em oleaginosas. Adaptado de Alu'datt et al. (2017).

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CAPÍTULO 2

PRIMEIRO ARTIGO

Bioaccessibility of defatted cashew nut kernel flour compounds released after simulated *in vitro* human gastrointestinal digestion

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Abstract

This study aimed to evaluate the effect of human gastrointestinal digestion on the antioxidant capacity, DNA protection and potential prebiotic effect of defatted cashew nut kernel flour (DCF). The gastric (pepsin) and intestinal (pancreatin/bile extract) phases were carried out at pH 3 and 7 (37°C/2h). After digestion the soluble and insoluble phases were separated by centrifugation. The DCF and soluble digested fraction (Ds) were characterized by amino acid composition, molecular weight (MW) distribution (SE-FPLC), total phenolic compounds and antioxidant capacity assays. Both MW distribution and amino acid profiles are of utmost importance for peptides biological activity. The Ds showed 84.5% of peptides with MM<3kDa in MW distribution, with higher soluble protein content (46,70 %), suggesting higher protein hydrolyze. The phenolic compounds content in Ds was 4.5-fold higher than in DCF extract (7.97 against 1.77 mg gallic acid/g sample) and the best extraction occurred by methanolic solvent. In addition, the ORAC and ABTS assays showed higher Ds antioxidant in aqueous extract (526.0 ± 1.60 and 76.64 ± 1.58 as µmol Trolox Eq./ g sample, respectively). These results suggest that the released compounds act by both mechanisms, proton donation and single electron transfer. Furthermore, Ds promoted higher super-coiled band DNA preservation than DCF. On the other hand, the insoluble digested fraction (Di) did not stimulate the commercial probiotics bacteria evaluated. In conclusion, gastrointestinal enzymes changed the original structure of the flour components (DCF), either by hydrolysis of the original protein forming bioactive peptides or by releasing bioactive compounds, such as phenolics, previously entrapped in the food matrix. These soluble compounds became more available to interact with reactive species, increasing the antioxidant capacity. Moreover, the insoluble fraction showed potential prebiotic effect to *Bifidobacterium lactis* BB-12.

Keywords: gastrointestinal digestion; *Anacardium occidentale L.*; DNA protection; antioxidant peptides; amino acid profile.

1. Introduction

Worldwide actions have been done aiming to address sustainable development, food security, human health and environmental preservation, as the 2030 Agenda for Sustainable Development and the new Circular Economy Action Plan for the European Union (UN, 2015; EU, 2020). Therefore, the biorefineries have been developed in order to extract biocomponents and convert them into more valuable products, contributing to reduced environmental impact and to build a more competitive and sustainable agribusiness (Contreras et al., 2019).

Anacardium occidentale L. is native from Brazil and it is the most widely known genus of *Anacardiaceae* family species, due to its cashew nut nutritional and economic value (Shahidi & Zhong, 2015). Industrial processing of cashew nut may generate about 40% of broken kernels which commercial value is much lower than the whole ones (de Carvalho, de Figueiredo, de Sousa, de Luna, & Maia, 2018). As cashew nut kernel profitability depends on the kernel extraction without breaking or damaging other derived by-products such as flour, edible oil and butter have been studied as food ingredients in order to add value to broken kernels (Oliveira, Mothé, Mothé, & de Oliveira, 2020).

The literature has described a variety of nuts and edible seeds compounds related to antioxidant, anti-inflammatory capacity, including the gut microbiota remodeling capacity (Sugizaki & Naves, 2018).Tree nuts are characterized as a source of nutrients like polyunsaturated fatty acids (PUFA), plant proteins, vitamins, minerals, fiber and polyphenols with high antioxidant potential (Sanchiz et al., 2019). Phenolics, oligosaccharides and PUFA have been describe as prebiotic, once they selective stimulate the host microorganisms proliferation (Gibson et al., 2017).

Antioxidant compounds have been extensively studied as prevention against oxidative stress and noncommunicable diseases (NCD), such as diabetes mellitus, some cancers, neurodegenerative and coronary heart disease (Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015). On the other hand, prebiotic expanded usage is based on the beliefs that modern day humans do not ingest sufficient quantities of lactic acid bacteria (LAB) or because its growth is stimulated by saccharolytic and/or proteolytic fermentation of resistant starch, non-digestible carbohydrates, oligosaccharides, proteins and mucins associated with physiological net benefits (Pineiro et al., 2008; Sousa et al., 2015). According to the ISAPP consensus statement, prebiotic effect is “a selectively stimulate bifidobacterial, lactobacilli or other species beyond these genus, evoking a measurable net benefit to host health, distinct from a control” (Gibson et al., 2017).

In vitro digestion is a widely employed method to predict gastro-intestinal food behavior and it represents a lower cost, faster, resource efficient and has no ethical restrictions compared to human trials (Minekus et al., 2014). The *in vitro* gastrointestinal digestion has been used to predict bioaccessibility of food biocompounds (Cilla, Bosch, Barberá, & Alegría, 2018).

Therefore, the main research work objective was to evaluate the impacts of defatted cashew nut kernel flour *in vitro* human gastrointestinal digestion on compounds digestibility, antioxidant capacity and a potential prebiotic effect of soluble fraction and insoluble fraction respectively.

2. Materials and Methods

2.1. Materials

Cashew nut kernels were acquired in the city of Fortaleza (CE/Brazil). Isolated commercial culture of probiotic bacteria was supplied by Sacco (*Lactiplantibacillus plantarum* BG112 and *Bifidobacterium animalis* BLC1) and Christian-Hansen (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* subspecie lactis BB-12). Chemicals and enzymes were supplied by Sigma-Aldrich (α-amylase from porcine pancreas A3176, pepsin from porcine gastric mucosa P7000, pancreatin from porcine P1750, and porcine bile extract B8631, 2,2'-Azobis(2-amidinopropane)-dihydrochloride granular (AAPH), fluorescein sodium salt, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-difenil-1-picrilidrazil (DPPH), 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxilic acid (Trolox), gallic acid), Thermo Scientific (SYBR safe), Dinâmica ($(NH_4)_2C_6H_6O_7$; $MnSO_4.H_2O$; K_2HPO_4;) KASVI (agar, yeast extract, beef extract, peptone bacteriological), Acumedia (peptic digest of animal tissue, peptone A), Synth ($NaC_2H_3O_2.3H_2O$; $MgSO_4.7H_20$, folin-ciocalteu reagent, n-hexane, ethanol, methanol, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, NaOH, HCl, $CaCl_2(H_2O)_2$), Neon (Polysorbate 80), Invitrogen (Ultrapure agarose). Na_2CO_3, 2,2,2-trichloroacetic acid (TCA), sodium phosphate buffer, TE buffer, TAE buffer.

2.2. Methods

2.2.1. Sample preparation

Cashew nut kernels were reduced in size in a food processor (Skymsen) and arranged inside hydraulic press (Carver laboratory press, model C) maintained at 9 kgf/cm^2 pressure for 24 hours, to maximum oil extraction. The semi defatted cake was grinded at the same food processor before a second oil extraction by n-hexane solvent in a Soxhlet

system, during 16 hours. This material, reduced to 5 mm size-particle, was the defatted cashew nut kernel flour (DCF).

Total phenolic compounds and antioxidant capacity by ORAC, ABTS and DPPH assays were determined in DCF and in the freeze-dried soluble digested fraction (Ds), generated after *in vitro* gastrointestinal digestion (GID). The DFC and Ds extracts were prepared as previously described by Kim, Jeong and Lee (2003), with some modifications. In summary, aiming to investigated the best fluid among deionized water, aqueous ethanolic solution (70:30 v/v) or aqueous methanolic solution (70:30 v/v) to solubilize bioactive compounds, 100 mg of samples (DCF or Ds) were added of the tested fluid (4 mL) and one-minute mixed through Ultra-Turrax T-25, followed by centrifugation (1125 x g, 10 minutes, 4 ºC). Then the supernatant was filtered in Whatman nº 2 paper into a 10 mL volumetric flask and the extraction was repeated once. The volumetric flask meniscus was completed with the same fluid and slit into eppendorfs, which were stored at -10º C. This procedure was done to each solvent and sample. These main extracts were tested in the assays cited above, adjusting the extract dilution, if necessary.

2.2.2. Physicochemical composition and phenolic compounds

Physicochemical compositions: moisture, lipids, total dietary fiber, protein, ashes were performed according to AOAC methods (Latimer & George, 2012) and carbohydrates were calculated by difference. Amino acid profile was carried out according to White, Hart and Fry (1986). In short, samples were hydrolyzed (HCl; 6 M; 110º C; 20 h) and it was conducted a derivatization in a high pressure liquid chromatograph, HPLC (Shimadzu Corporation, Kyoto, Japan), with a reverse-phase column (LUNA C18, 100- 5 µm, 4.6 mm x 250 mm) (Phenomenex, Torrance, CA, USA). Amino acids were quantified by comparison with amino acids standard Thermo Scientific (Rockford, IL, USA) and DL-2-aminobutyric acid (Sigma-Aldrich®, St.Louis, MO, USA) was used as an internal standard. The amino acid score was determinate according to WHO/FAO/UNU (2007).

Phenolic compounds quantification was done in aqueous, ethanolic and methanolic extracts (as described in the 2.2.1 item) based on the aromatic rings oxidation by Folin-Ciocalteau reagent (Al-Duais, Müller, Böhm and Jetschke, 2009). It was performed also assays which had the main extract added of 2,2,2-trichloroacetic acid (TCA) whereby soluble aromatic amino acid interference was reduced. So 300 µL of the original extract (aqueous, ethanolic and methanolic) was mixed to 60 µL TCA 10% (w/v), incubated at 4ºC by 10 minutes and centrifugated (17,949 x g, 5 minutes). These supernatants were applied to phenolic compounds protocol.

2.2.3. SE-FPLC molecular weight (MW) distribution

DFC and Ds samples were solubilized (5 mg / mL) in sodium phosphate buffer (25 mM pH 7,4 with 150 mM NaCl), sonicated for 10 minutes and filtered in 45 µm polytetrafluoroethane membrane before injection (500 µL). The isocratic elution in flow 0,5 mL / minutes was performed at Akta Pure chromatography (GE Healthcare, Chicago, Illinois, EUA) with Superdex 30 column during 60 minutes and UV-Vis 280 nm detector. The standards α-lactalbumin (14,178 Da), Insulin (5,807.6 Da), Vitamin B12 (1,355.37 Da) and L-β-4-dihydroxyphenylanine (197.2 Da) were used to build an analytical curve, from which it was possible to establish MW ranges according to the retention time of aromatic compounds (Vander Heyden, Popovici, & Schoenmakers, 2002).

2.2.4. Simulated human gastrointestinal digestion *in vitro*

The *in vitro* gastrointestinal digestion was done according to Minekus et al. (2014), in water bath at 37 ºC under stirring (90 rpm). Briefly, DCF (5 g) was added of deionized water (20 mL) prior to the simulated salivary fluid (SSF), aiming to enable α-amylase (75 U / mL) dispersal in oral phase (pH 7, 10 minutes). In succession, the pH was lowered to 3.0 through HCl 6 M addition, followed by simulated gastric fluid (SGF) and pepsin (2.000 U / mL) incorporation. The gastric phase was conducted for 120 minutes and after that, the intestinal phase started by pH adjustment to 7.0 based on NaOH 1 M insertion. It was also inserted the simulated intestinal fluid (SIF) and enzymes (porcine pancreatin (100 U / mL) and porcine bile extract (10 mM)), developing this stage for 120 minutes. Four trials of the simulated *in vitro* gastrointestinal digestion (GID) were performed. In order to stop the hydrolysis, the total digested volume was heated up to 90 ºC by 15 minutes, cooled down to 4 ºC and centrifuged (3,645 x g, 30 minutes, 4 ºC). The digested soluble (Ds) and insoluble (Di) fractions were collected and freeze-dried. The same protocol was conducted without sample additions (blank), to be discounted from physicochemical composition sample results.

2.2.5. Antioxidant assays

2.2.5.1. ORAC

Oxygen radical absorbance capacity, ORAC, was measured by the peroxyl radical (ROO•) scavenger capacity to protect the fluorescein molecule from oxidation, as describe by Chisté, Mercadante, Gomes, Fernandes, Lima and Bragagnolo, (2011). The assay was carried out in a 96-wells microplate fluorescence reader (Synergy, BioTek®, software Gen5), with fluorescence filters for excitation at 485nm and for emission at 528 nm at 37 ºC. A trolox standard curve was used to express samples ORAC values. The samples and stander net

protection (AUC_{net}) was calculated by the difference between the area under the fluorescence decay curve of samples/stander ($AUC_{sample/stander}$) and the area under the fluorescence decay curve without sample or Trolox addition (AUC_{blank}). The results were expressed in µmol Trolox equivalent/g sample.

2.2.5.2. ABTS

The antioxidant assay measured the ABTS radical scavenging capacity according to Al-Duais, Müller, Böhm and Jetschke (2009). Shortly, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) radical solution was done by mixing 88 µL aqueous persulfate potassium ($K_2S_2O_8$) 140mM and 5 mL of aqueous ABTS solution 7mM. The mixture remained in darkness for 16 hours and had its absorbance adjusted to 0.7 ± 0.02 at 734 nm in UV-Vis spectrophotometer just before the analysis. A 20 µL aliquot of each extract were put into a microplate well and 220 µL of ABTS working solution (Abs 0.7 ± 0.02) was added to react during 6 minutes. After that, the absorbance was read at 730nm in UV-Vis microplate Synergy reader (BioTek®, software Gen5). A trolox standard curve in phosphate buffer 75 mM and pH 7.4 was used to express ABTS values. The absorbance of 240 µL of sodium phosphate buffer (75 mM, pH 7.4), Abs_{blank}, was discounted from samples and standard absorbance and by the analytical curve, final results were expressed as µmol Trolox equivalent / g sample.

2.2.5.3. DPPH

2,2-Diphenyil-picrylhydrazyl (DPPH) radical scavenging capacity was performed in 96-well UV-Vis microplate Synergy reader (BioTek®, software Gen5) also according to Al-Duais, Müller, Böhm and Jetschke (2009). In each sample well, 134 µL of ethanolic DPPH solution 150µM was added to 66 µL of extract. The reaction mixture was kept in darkness for 45 minutes before the absorbance reading at 517 nm. The control was carried out by the addition of 66 µL of ethanol instead of the sample and the blank by 200 µL of ethanol only. A trolox standard curve in ethanol was made and the antioxidant capacity was calculated as µmol trolox equivalent/g sample.

2.2.5.4. DNA protection capacity

Plasmidial DNA pcDNA-FLAG (Pavan et al., 2016) was used in a 0.8% agarose gel electrophoresis to evaluate DFC and Ds aqueous extract antioxidant capacity against peroxyl radical, generated by AAPH thermal degradation (Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015). In order to eliminate any intrinsic proteolytic enzyme, the DCF

was heated at 90° C by 10 minutes. In a DNAse free microtube, the following reagents were added at this stated order: 4 µL of DNA plasmidial supercoiled pcDNA (125 ng / mL) prepared at Tris-EDTA buffer, 2 µL of samples extract, 4 µL of 30 mM aqueous APPH solution. In order to have a DNA supercoil band intensity control for native DNA form (positive control), 6 µL of deionized water were added instead sample and AAPH. Also a DNA supercoil band intensity control for oxidated DNA form (negative control) was preformed, by adding 2 µL of deionized water instead the sample volume. All these four treatments were incubated in darkness at 37° C for 1 hour. The reaction mixture (10 µL) was applied in a 0.8 % agarose gel electrophoresis using Tris-acetic acid ETDA buffer, running at 80 mV for 1.5 hours followed by 120 mV during 1 hour. The gel was stained with 1:20.000 SYBR safe (Thermo Scientific) added to TAE-buffer and then the bands were visualized under UV light at ChemiDoc Imaging System (Bio-Rad). The quantification was done in Image J software. The results were expressed as following equation (1).

$$Retention\ supercoiled\ DNA\ band\ (\%) = \frac{intensit\ of\ sample\ supercoiled\ band}{intensit\ of\ control\ supercoiled\ band} * 100$$

2.2.6. Potential prebiotic effect

The prebiotic effect was conducted as prior settled by Moreno-Vilet et al. (2014), evaluating four commercial probiotic strains growth *Lactobacillus plantarum* BG112 (recently renamed as *Lactiplantibacillus plantarum* BG112 according to Zheng et al. (2020)), *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BLC1 and *Bifidobacterium animalis* subsp. *lactis* BB-12) in De Man-Rogosa-Sharpe (MRS) medium that had replaced its carbohydrate source (dextrose) for the digested insoluble fraction (Di, 3.77g). As growth controls, these bacteria were also inoculated in four other MRS media: with any carbohydrate source (negative control, MRS_{nc}), with the standard dextrose content (positive control, MRS_{pc}, 4g dextrose), with adjusted dextrose content (adjusted positive control MRS_{apc}, 1.89 g dextrose) and with inulin as carbohydrate source instead dextrose (inulin control., MRC_{inulin}, 1.89 g inulin). The MRS_{pc} had in its formulation sodium acetate (1g), agar (3g), dibasic ammonium citrate (0.4g), peptic digest of animal tissue (peptone A, 2g), beef extract (2g), yeast extract (1g), potassium phosphate (0.4g), magnesium sulfate (0.02g), manganese sulfate (0.01g), 200 µL of polysorbate 80 and dextrose (4g) diluted in 200 mL of deionized water. The MRS_{apc} and MRS_{inulin} were prepared to have the same carbohydrate amount of Di (calculated by physicochemical composition). All the five MRS mediums were autoclaved. Freeze-dried commercial probiotic bacteria were resuspended in peptone water 0.1% (w/v) and 100 µL were inoculated in each plate. The *L. acidophillus*

and *L. plantarum* were incubated in microaerophilia mainwhile *Bifidobacterium sp* were kept in anaerobiosis, ensured by the use of AnaeroGen*,* both for 72 hours at 37 ºC. After the incubation time, spread plate technique was used for plate counting and the results were expressed as log cfu / g. All probiotics and medium where compared to the MRS_{nc}.

2.3. Statistical analysis

The results were expressed as means ± SD (standard deviation) of three measurements. It was applied one-way ANOVA with Tukey test and t-Test, at a significance level of $p<0.05$. The exception were DNA protective capacity assay and potential prebiotic effect, that have been evaluated by one-way ANOVA and Dunnet test (also at a $p<0.05$ significance level). The statistical program was GraphPad Prism 8.0.1.

3. Results and Discussion

3.1. Physicochemical composition and total phenolic compounds

Through the digestive enzymes hydrolysis, 62.64% of the initial material ware solubilized, since from 4.40 ± 0.01 g DCF resulted into 2.69 ± 0.39 g of digested soluble fraction (Ds, 61.19%) and 1.67 ± 0.39 g of digested insoluble fraction (Di, 37.96%) after freeze drying, expressing in dry matter. Physicochemical composition (Table 1) were analyzed to characterized the samples before and after *in vitro* digestion.

According to Table 1, plant protein is the major macro nutrient from all samples and 69.71% became soluble in Ds (1.25 ± 0.18 g) after *in vitro* digestion. Once the macronutrients is solubilized them became more bioaccessible and it’s an important prior condition to biodisponibility and bioactivity (Cilla et al., 2018). The Di fraction presents protein and dietary fibers, which may stimulate bacteria proliferation (Gibson et al., 2017).

Table 1. Physicochemical composition of cashew nut kernel' samples, expressed in dry matter.

Components (%)	SCF	DCF	Ds	Di
Lipids	28.29 ± 0.28 $^{A, b}$	0.76 ± 0.15 $^{B, d}$	nd	nd
Protein	30.43 ± 0.39 $^{C, a}$	40.74 ± 0.01 $^{B, a}$	46.70 ± 0.04 $^{A, a}$	40.41 ± 0.07 $^{B, a}$
Ashes	3.68 ± 0.01 $^{D, d}$	4.64 ± 0.01 $^{C, c}$	15.54 ± 0.05 $^{A, b}$	11.29 ± 0.03 $^{B, c}$
Carbohydrate	37.60	53.86	37.76	48.30
Total dietary fiber	8.55 ± 0.13 $^{C, c}$	11.45 ± 0.18 $^{B, b}$	un	16.26 ± 0.40 $^{A, b}$

Values are means ± standard deviations of three (3) measurements. Carbohydrate was determinate by difference. Abbreviation includes SDF: semi defatted flour; DF: defatted flour; Ds: soluble digested fraction; Di: insoluble digested fraction; nd: not determined; un: undetected < 0.10%. Different superscript capital letters in the same row and superscript small letters in the same column are significantly different at $p<0.05$.

Based on the amino acids score for adult's protein requirement (WHO/FAO/UNU, 2007) and amino acid composition in DCF and Di (Table 2), the cashew nut's protein can be classified as a source of good nutritional protein, containing all indispensable amino acid with low score just for lysine. The DCF showed high score for phenylalanine and tyrosine but low of lysine, a profile similar to legumes. The Ds presented reduced concentration of sulfur amino acids, influenced by methionine reduction (39.11%) and phenylalanine (15.51%). Both of them have been described as an antioxidant, amongst with tyrosine, tryptophan, cysteine, and histidine (Ohashi et al., 2015). The hydrophilic amino acids are the majority group for both DCF and Ds, despite the hydrophobic ones have been describe as antioxidants (Sarmadi & Ismail, 2010).

Table 2. Amino acids profile from defatted cashew nut kernel flour (DCF) and defatted cashew nut kernel digested soluble fraction (Ds). The chemical score was calculated by WHO/FAO/UNU (2007)[a] protein reference for adults (> 18 years).

Amino acid (AA)	WHO/FAO/UNU[a] mg / g protein	DCF mg / g protein	DCF AA score	Ds mg / g protein	Ds AA Score
Indispensable					
His	15	22.32 ± 0.05	1.49	22.42 ± 0.65	1.49
Ile	30	37.89 ± 0.04	1.26	39.30 ± 0.05	1.31
Leu	59	72.93 ± 0.35	1.24	64.37 ± 0.05	1.09
Lys	45	41.85 ± 0.37	0.93	43.62 ± 0.03	0.97
Met + Cys	22	21.11 ± 0.06	1.14	17.99 ± 0.05	0.82
Phe + Tyr	38	82.21 ± 0.10	2.16	74.74 ± 0.08	1.97
Thr	23	36.34 ± 0.15	1.58	33.70 ± 0.03	1.47
Trp	6	4.80 ± 0.05	0.80	7.76 ± 0.10	1.29
Val	39	53.95 ± 0.37	1.38	52.88 ± 0.17	1.36
Dispensable					
Asp		92.68 ± 0.41	np	105.73 ± 0.08	np
Glu		219.67 ± 0.95	np	233.05 ± 0.22	np
Ser		58.40 ± 0.24	np	55.10 ± 0.08	np
Arg		127.11 ± 0.60	np	124.00 ± 0.08	np
Ala		42.43 ± 0.13	np	38.31 ± 0.13	np
Pro		39.88 ± 0.01	np	37.41 ± 0.11	np
Gly		47.22 ±0.19	np	49.63 ± 0.22	np
Distribution (%)					
Hydrophobic		31.62		29.38	
Hydrophilic		50.37		52.87	
Neutral		18.01		17.75	

Hydrophobic (Ala, Val, Met, Phe, Leu, Ile, Pro, Trp) Hydrophilic (Arg, Asp, His, Lys, Glu) Neutral (Ser, Gly, Thr, Tyr, Cys)* Asp = Aspartic acid, Ala = Alanine, Arg = Arginine, Gln = Glutamine, Gly = Glycine, His = Histidine, Cys= Cysteine, Ile = Isoleucine, Leu = Leucine, Lys = Lysine, Met = Methionine, Phe = Phenylalanine, Pro = Proline, Ser = Serine, Thr = Threonine, Glu = glutamate, Trp = Tryptophan, Tyr = Tyrosine, Val = Valine. Values are means ± standard deviations of three (3) measurements. np: not applicable.

Phenolic compounds (Table 3) were selectively extracted according to the solvent used and some of them widely used are water, ethanol and methanol (Azmir et al., 2013). Bodoira and Maestri (2020) described phenolics as soluble compounds in polar solvents (water), low molecular weight alcohols (methanol, ethanol) and organic solvents. At DCF samples, there were no difference among the solvents efficiency, although the TCA application reduced the proteins and large peptides interference in water extraction (Greenberg & Shipe, 1979). The concentration of hydroxyl groups in water helps on phenolics solubilization (glycosides more than aglycones form) (Bodoira & Maestri, 2020).

The *in vitro* digestion could be able to isolated bioactive compounds, which were quantified based on their solubility (Cilla et al., 2018) since Ds fraction showed higher phenolic concentration than DCF in all solvents tested despite methanol extraction had had the highest efficiency. The methanol extract also proteins, once the TCA application reduced the phenolic compounds quantified by Folin-Ciocalteau reagent oxidation (15.34 and 7.94 mg of gallic acid / g of sample, without and with TCA, respectively).

Chandrasekara and Shahidi (2011) have obtained 1.14 ± 0.43 and 0.028 ± 0.01 of GAE mg/g of defatted cashew kernel meal for soluble and bound total phenolics. This value is close to ethanolic and aqueous extracts with TCA, important to minimize aromatic amino acids interference. Sanchiz et al. (2019) have demonstrated the phenolic content in unprocessed nuts, including cashew nut. They founded anthocyanins, flavonols and tartaric esters (5 ± 2 µg cyanidin/g DW, 33 ± 2 µg quercitin/g DW and 132 ± 8 µg caffeic acid/g DW, respectively).

Table 3. Total phenolic compounds in different solvents and both treatments (with and without 10% TCA addition).

	Phenolic Compounds (mg GAE /g sample)					
	Without TCA			**With TCA**		
Extract / Sample	**Aqueous**	**Ethanolic**	**Methanolic**	**Aqueous**	**Ethanolic**	**Methanolic**
DCF	2.09±0.07 $^{b, A}$	1.85±0.06 $^{b, AB}$	1.76±0.09 $^{b, AB}$	1.05±0.15 $^{b, C}$	1.44±0.37 $^{b, BC}$	1.77±0.07 $^{b, AB}$
Ds	8.31±0.04 $^{a, B}$	7.04±0.18 $^{a, BC}$	15.34±0.06 $^{a, A}$	6.90±0.69 $^{a, BC}$	5.21±0.81 $^{a, C}$	7.97±1.27 $^{a, B}$

Values are means ± standard deviations of three (3) measurements. Different small letters on the same column are different by t-test (p ≤ 0.05). Different capital letters on the same row indicate difference by one-way ANOVA (p ≤ 0.05). DCF: defatted cashew nut kernel flour; Ds: defatted cashew nut kernel flour digested soluble fraction.

Among the solvents tested, methanol was described on the literature as the most efficient one, able to extract anthocyanin, saponins, tannins, terpenoids polyphenols and flavones. Water can solubilize just anthocyanin, saponins, tannins and terpenoids. On the other hand, ethanol is able to extract tannins, terpenoids, polyphenols, flavonol and alkaloids (Azmir et al., 2013). Although methanol presents higher efficiency, in food applications ethanol should be preferred due to methanol toxicity (Ignat, Volf, & Popa, 2011).

Bodoira and Maestri (2020), claimed that key cashew nut kernel phenolics are flavonols (catechin and epicatechin and their gallic acid esters), and proanthocyanidins, but it also has phenolic lignans as minor compounds and a specific category of phenolics characteristic from Anacardiaceae family plants called alkylphenols (cardanols, cardols,

anacardic acid and their derivatives). These alkylphenols (lipophilic compounds) have been reported to develop reactive oxygen species (ROS) scavengers and strong evidence of superoxide anion and uric acid inhibit generation by xanthine oxidase (Bodoira & Maestri, 2020). Once these phenolics are major lipophilic phenolics their concentration at defatted cashew nut flour is reduced. Further, phenolic compounds exist in free and bounded form in a food matrix, and different writers have already discussed their antioxidant capacity (Alu'datt et al., 2017).

3.2. Molecular weight (MW) distribution

The molecular weight (MW) distribution profile by SE-FPLC is shown in Figure 1 and revels the reduction on compounds MW, at the analysis condition. Compounds which had MW<3 kDa represents 23.07% of DCF soluble compounds but 83.25% of the Ds soluble compounds. It's possible to infer that gastrointestinal enzymes showed effectiveness to increase the total DCF's soluble compounds and to reduce the molecular weight (MW). It should be noted that the detector was limited to the wavelength of 280 nm, consequently, it was only possible to identify aromatic compounds with no distinction between peptides and phenolic compounds, for example (Albe Slabi et al., 2019).

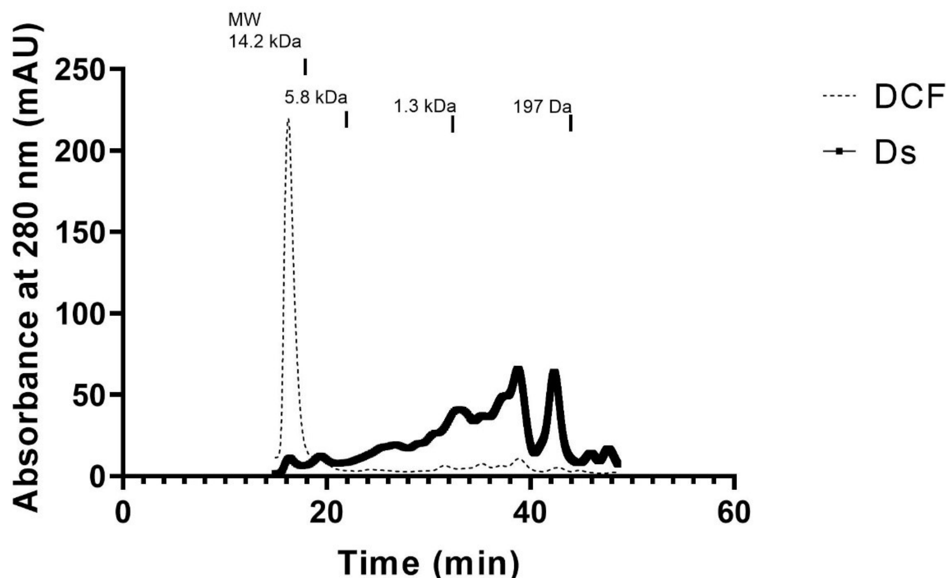

Figure 1. Molecular weight (MW) distribution profile by SE-FPLC of defatted cashew nut flour (DCF; dashed line) defatted cashew nut digested soluble fraction (Ds; continuous line).

Prior studies have described DCF albumins and globulins protein as nine polypeptides chains that have estimated MW of 57.54 kDa, 55.21 kDa, 51.29 kDa, 29.17

kDa, 23.99 kDa, 22.91 kDa, 16 kDa, 12.88 kDa and 10 kDa. Besides that glutelins (12,000-22,000 Da) and prolamins (26.300 Da) have one polypeptide (Liu, Peng, Zhong, Liu, Zhong & Wang, 2018; Sathe, Venkatachalam, Sharma, Kshirsagar, Teuber, & Roux, 2009). That explains the higher chromatographic peak around 15 minutes and the largest area of the compound with MW> 7 kDa in DCF, according to Table 4.

Table 4. DCF and Ds' molecular weight distribution area percentage and absolute values.

MW interval (kDa)	DCF		Ds	
	MW area percentage (%)	Total area	MW area percentage (%)	Total area
> 7	72,43	300,89	6,95	52,62
5-7	1,68	6,97	2,71	20,50
3-5	2,83	11,76	7,10	53,74
1-3	6,73	27,97	25,05	189,65
0,1-1	16,33	67,86	58,20	440,63
Total	100,00	415,44	100,00	757,14

Abbreviations include: DCF: defatted cashew nut kernel flour; Ds: defatted cashew nut kernel flour digested soluble fraction.

3.3. Antioxidant capacity – ORAC, ABTS, DPPH and DNA supercoil band protection

At the analysis conditions, Ds presented higher antioxidant capacity than DCF for ORAC and ABTS assays, in all solvent tested. Nonetheless, Ds antioxidant capacity was smaller at DPPH assay in aqueous extract and was not detected in ethanolic and methanolic extracts. The ORAC, ABTS and DPPH assay results are shown at Table 5.

The DCF showed high antioxidant activity by ORAC assay in ethanolic and methanolic extracts. Furthermore, ORAC aqueous extract did not show difference from ABTS and DPPH, regardless of the solvent used. The aqueous methanolic (70:30 v/v) extraction was more effective and developed higher ORAC activity than methanolic extraction in Sanchiz et al. (2019) study. After GID, the solubility of the compounds changed. The highest antioxidant capacity was noticed to ORAC aqueous extract, followed by methanolic and ethanolic ones. The soluble compound showed preference for ABTS radical scavenging than DPPH radical.

Some antioxidant mechanisms for radical and ROS scavenger have been described and the most accepted are hydrogen atom transfer, HAT, (such as oxygen radical absorbance capacity (ORAC) and inhibition of lipoperoxidation) and single electron transfer (SET) (for example ferric reducing ability of plasma (FRAP), Folin-Ciocalteu's phenol reagent reducing capacity, radical scavenging effects related to 1,1-diphenyl-2-

picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals) (Granato et al., 2018; Shahidi & Zhong, 2015). As a result, is possible to infer that DCF and Ds have both HAT and SET antioxidant capacity, highlighting the Ds antioxidant capacity by HAT mechanism.

Plant foods are potential sources of dietary antioxidants (for example vitamin C, α-tocopherol, carotenoids, flavonoid and phenolic acids) capable to prevent oxidative damage from pro-oxidant species, reactive oxygen species, ROS (such as superoxide radical ($O^{\bullet}{}_2^-$), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$)) or non-free-radical species (hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3)) (Gülçin, 2012). Reactive oxygen species (ROS) can lead to DNA damage and initiate certain carcinogenesis or chronic diseases (Zhang, Mu, & Sun, 2014). Dietary antioxidants help endogenous antioxidant defense, composed by enzymatic (superoxide dismutase and glutathione peroxidase) and non-enzymatic (antioxidant vitamins, trace elements, coenzymes and cofactors) mechanisms to re-establish the balance between pro-oxidant-antioxidant, reducing the oxidative stress condition (Sarmadi & Ismail, 2010). Oxidative stress *in vivo* causes cellular destruction and apoptosis by oxidation of cellular membranes, protein, enzymes and DNA (Shahidi & Zhong, 2015).

Another approach to determinate the protein value is the ability to generate bioactive peptides and a large number of peptides have been described as antioxidants (Kitts & Weiler, 2003). Peptides with sulfur-containing amino acids, aromatic amino acids and histidine play important roles in the antioxidant activity (Ohashi et al., 2015). These peptides contain from 5 to 16 amino acid residues (Sarmadi & Ismail, 2010). According to amino acid profile (Table 2), the higher concentration of cysteine, tryptophan, tyrosine and histidine and the lower MW of soluble compounds at Ds can infer the presence of potential antioxidant peptides.

Table 5. Antioxidant capacity by aqueous, ethanolic and methanolic extracts at ORAC, ABTS and DPPH assay. The results are expressed as µmol Trolox Eq./ g sample.

Assay (µmol Trolox Eq./g sample)	Extract	DCF	Ds
ORAC	Aqueous	14.8 ± 0.02 $^{b, B}$	526.0 ± 1.60 $^{a, A}$
	Ethanolic	46.3 ± 1.22 $^{a, B}$	101.3 ± 4.70 $^{c, A}$
	Methanolic	39.7 ± 7.64 $^{a, B}$	203.2 ± 13.58 $^{b, A}$
ABTS	Aqueous	5.27 ± 0.12 $^{b, B}$	76.64 ± 1.58 $^{d, A}$
	Ethanolic	10.57 ± 0.61 $^{b, B}$	57.81 ± 5.87 $^{de, A}$
	Methanolic	6.68 ± 0.78 $^{b, B}$	53.32 ± 1.29 $^{e, A}$
DPPH	Aqueous	4.77 ± 0.60 $^{b, A}$	3.61 ± 2.43 $^{f, A}$
	Ethanolic	6.76 ± 0.13 ^{b}	-
	Methanolic	7.35 ± 1.72 ^{b}	-

Values are means ± standard deviations of three (3) measurements. DCF: defatted cashew nut kernel flour; Ds: defatted cashew nut kernel flour digested soluble fraction. (-): not detected. Different small letters in the same column indicate difference by one-way ANOVA ($p \leq 0.05$). Different capital letters in the same row indicate difference by t-test ($p \leq 0.05$)

The oxidation of DNA molecules induced by AAPH leads to its breakage, releasing two parts, one circular and another linear form (Yarnpakdee, Benjakul, S., Kristinsson, H. G., & Bakken, 2015), as shown in Figure 2. Through the comparison of DNA supercoil band among samples, the DNA intensity with DCF addition was as low as the DNA+APPH assay. However, the DNA intensity was retained by 29,02% with Ds. Although soluble compounds in Ds promoted DNA supercoil band protection, it's still different from the DNA control. Therefore, it is not possible to associate an effective DNA protective capacity to Ds.

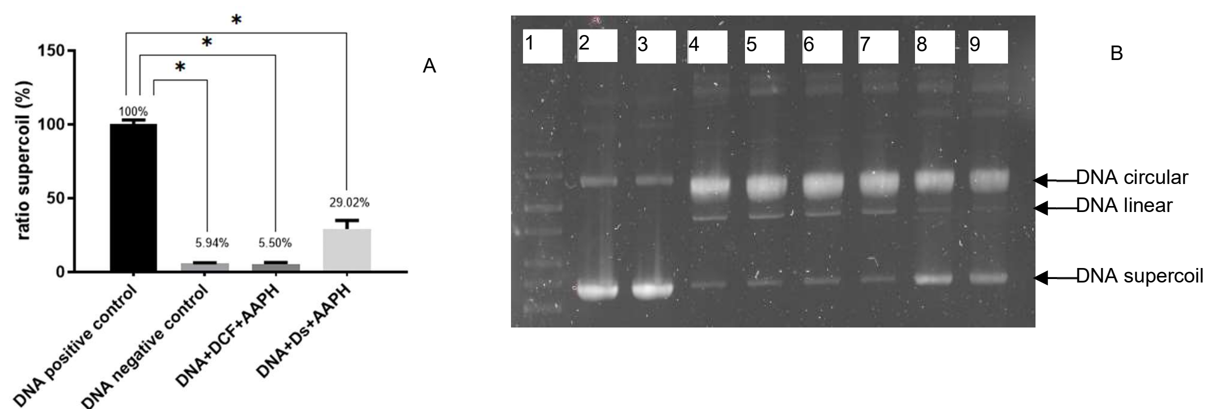

Figure 2. DNA Protection assay: (a) DNA supercoil band protection capacity with the four treatments: DNA positive control, DNA negative control, DNA+AAPH+DCF and DNA+AAPH+Ds. Samples with (*) are different from DNA supercoil control; (b) agarose gel electrophoresis which numbers refer to 1: Molecular weight

standard; 2 and 3: DNA positive control; 4 and 5: DNA negative control; 6 and 7: DNA+AAPH+DCF; 8 and 9: DNA+AAPH+Ds.

3.4. Potential prebiotic effect

As a result of probiotic growth stimulation analysis showed in Figure 3, *Bifidobacterium animalis* subsp. *lactis* (BB-12) was positively stimulated by Di fraction, when compared to negative control (MRSnc, without carbohydrate addition), developing a potential prebiotic effect. Different from Moreno-Vilet et al. (2014), who had describe a growth between 8 and 8.5 log cfu/g in MRS medium without carbohydrate source, at the present research, all strains tested had a high proliferation (from 11.00 to 11.91 log cfu/g) in this control. Subtracts that might affect microbiota composition an metabolic composition for different mechanisms that the selective utilization by the host microorganism are not considered as prebiotic and some of these compounds are proteins and fats, probiotics, antibiotics, minerals, vitamins and bacteriophages (Gibson et al., 2017). It might be a limitation for this method application, which could not show difference among LA-5, *L. plantarum* and BCL-1 bacteria growth and their respective MRS_{nc} plate, since it has nitrogen source on its composition. Phylogenetic differences between bacteria may reflect their ability to use carbohydrate source as a growth factor (Moreno-Vilet et al., 2014).

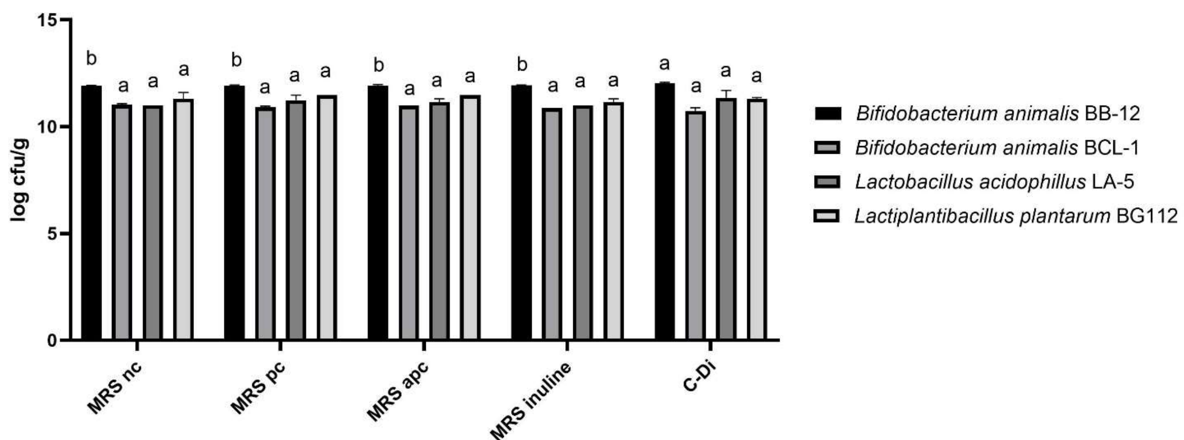

Figure 3 Prebiotic assay. Abbreviations include: defatted cashew nut kernel flour digested insoluble fraction added in MRS media as the only carbon source (C-Di), negative control (MRSnc): didn't have any carbon source added. Standard positive control (MRSpc) and adjusted positive control (MRSapc) had dextrose addition and prebiotic control (MRSinulin), had inulin added. Values are means ± standard deviations of two measurements. Values with different superscript letters are significantly different at $p<0.05$ to the MRSnc.

The inulin and short-chain FOS have been described as good oligosaccharides for stimulating acid lactic bacteria (Anadón, Castellano, & Martínez-Larrañaga, 2014). On the basis of the extensive available literature, the current candidates as well accepted prebiotics are lactulose, fructooligosaccharides (FOS), galactooligosaccharides (GOS) conjugated linolenic acid (CLA) and polyunsaturated fatty acid (PUFA), oligosaccharides (e.g., mannanoligosaccharide (MOS), xylooligosaccharide (XOS), soya-oligosaccharides (SOS), gluco-oligosaccharides, isomalto-oligosaccharides (IMO), human milk oligosaccharides (HMO)), lactosucrose, levans, pectic-oligosaccharides, resistant starch, sugar alcohols, phenolics compounds, phytochemicals and dietary fiber (Gibson et al., 2017; Anadón, Castellano, & Martínez-Larrañaga, 2014).

According to Sugizaki and Naves (2018) the nuts rich in polyphenols can modulate the microbiota and therefore present a prebiotic effect. Although anthocyanins, flavonols and tartaric esters were founded in cashew nut (Sanchiz et al., 2019). The prebiotics has been develop not only for antipathogenic effects (e.g, inhibiting adhesion of pathogenic organisms to the gut mucosa) but also to decrease fecal transit time, lower cholesterol and the glycemic response, improve bone health, lower daily energy (fat) intake, relieve symptoms of inflammatory bowel disease and attempt to lower colon cancer rates (Pineiro et al., 2008).

A food ingredient can be classified as prebiotic since it fulfills resistance to digestion (without hydrolysis or absorption in the upper intestine), good fermentation by the large intestinal microbiota and a selective effect on the microbiota that has associated health promoting effects (Anadón, Castellano, & Martínez-Larrañaga, 2014). The FAO technical meeting for prebiotic indicates the component characterization (source, origin, purity, chemical composition, structure), functional characterization *in vitro* or *in vivo* testing using animals as prior stages to product formulation, safety assessment and double blind, randomized and controlled human trials leading to their safe and efficacious use in food (Pineiro et al., 2008). Further analysis should be conducted in order to identify the potential prebiotic compound in the Di. The isolation of a specific cashew nut kernel compound may be a promising approach.

4. Conclusion

In conclusion, defatted cashew nut kernel flour (DCF) is a source of bioactive compounds. Their solubilization whereby gastrointestinal enzymes hydrolysis releases them from the DCF matrix. The antioxidant capacity after GID (in the Ds fraction) might be correlated to parent protein hydrolysis forming bioactive peptides or to phenolic compounds.

The insoluble matter (Di) had shown potential prebiotic effect, being advisable to deeply investigate isolated compound responsible for this selective bacteria growth stimulation.

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CAPÍTULO 3

SEGUNDO ARTIGO

In vitro models to evaluate the expression and bioaccessibility of bioactive compounds from sunflower and palm kernel meal.

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Abstract

Sunflower (*Helianthus annuus L.*) and palm tree (*Elaeis guineensis Jacq.*) are among the most cultivated oil seeds-crop worldwide. The edible oil industry disposals a huge amount of by-product, source of bioactive compounds. This study aimed to evaluate potential antioxidant and prebiotic compounds released after *in vitro* gastrointestinal digestion. Semi defatted sunflower and palm kernels were submitted to n-hexane extraction. Defatted sunflower flour (DSF) and pretreated defatted palm kernel flour (DPFa) were *in vitro* digested and the soluble (Ds) and insoluble (Di) fractions were separated by centrifugation. The phenolic compounds, molecular weight (MW) distribution profile and antioxidant capacity by several methods (DPPH, ABTS, ORAC and DNA protection) were determinate in sunflower's Ds (SDs) and palm kernel's Ds (PDs) extracts. The sunflower's Di (SDi) and palm kernel's Di (PDi) probiotic bacteria growth capacity were evaluated in MRS medium. The protein and total phenolic content were 52,41 ± 0,02 % and 20.10 ± 1.14 mg GAE / g sample besides 7.82 ± 0.13 % and 0.27 ± 0.01 mg GAE / g sample in DSF and DPF, respectively. The MW distribution showed 73.47% and 35.06% of compounds with MW<3kDa in SDs and PDs. The SDs and PDs presented higher antioxidant capacity than their original flours, showing 264.28 ± 2.19 and 4.83 ± 1.21 by DPPH and 187.37 ± 4.55 and 73.22 ± 1.60 by ABTS and 923.3 ± 28.01 and 280.8 ± 4.03 μmol Trolox eq. /g sample in ORAC assay (in methanolic extract). Both Ds preserved more the DNA supercoil band compared to the defatted flour and the DNA oxidized control, but just PDs showed DNA protective capacity. The Di fractions have not shown a potential prebiotic effect.

Keywords: *Helianthus annuus L.*, *Elaeis guineensis Jacq.*, phenolic compounds, DNA protection, prebiotic effect.

1. Introduction

The circular economy has been addressed as a solution for actual society challenges regarding economic growth, environmental resources scarcity and humanity survival (Geissdoerfer, Savaget, Bocken, & Hultink, 2017; Ghisellini, Cialani, & Ulgiati, 2016). In the agribusiness, the by-products valorization by the biorefineries concept can innovate the sector increasing its profitability, sustainability and competitivity by environmental impact reduction and value-added by-products constituent's separation (Contreras et al., 2019). The agro-industrial by-products have valuable biocomponents such as protein, polysaccharides, fibers and phytochemicals, that can be applied as food ingredients, with nutrition and pharmacological goal (Alexandre, Moreira, Castro, Pintado, & Saraiva, 2018).

A well-balance nutrition and adequate access to food are fundamental aspects to body development and growth, for body function maintenance beside to contribute to chronic degenerative diseases prevention (FAO,2013). Diet management aiming to control health disorders and gut microbiota modulation become an usual approach and move food industry forward functional product development (Vieira da Silva, Barreira, & Oliveira, 2016). Nowadays, the vegetarian and vegan publics also increased the demand for plant protein products (Hayes, 2018). The development of functional foods should be associated to compounds bioaccessebility and biodisponibility, previous conditions to bioactivity (Cilla, Bosch, Barberá, & Alegría, 2018).

The by-product resultant from sunflower and palm kernel oil extraction are mainly use in feed, regarding their insoluble fibers content. However, regarding to protein and plant secondary metabolic content (such as phenolic compounds), these by-products have a potential to be applied as food ingredients after properly treated (Schmidt & Pokorný, 2011). Phenolic compounds are reactive due to the presence of aromatic rings in their chemical structure, important to their biological action as antioxidants. Many studies have correlated phenolic compounds daily consumption to reduction in risk of chronic degenerative diseases

(Macedo, Battestin, Ribeiro, & Macedo, 2011). The actual trend is to maintain protein and phenolic compounds associated, due to their antioxidant capacity (Nazzaro, Fratianni, Ombra, D'Acierno, & Coppola, 2018).

Researches regarding food or food components with antioxidant properties use *in vitro* methods to predict their capacity. This approach makes possible to compare different food matrix and investigate their antioxidant capacity. Assays like ABTS, DPPH and ORAC tested with peptides have the main target to prospect the major potential to assist in the body antioxidant defense, considering the daily consumption as a dietary component (Tsai et al., 2011).

In conclusion, the by-product valorization as a bioactive molecules source is useful ether by environmental or economic point of view (Mirzaei et al., 2015). This study aimed to characterize major compounds on sunflower and palm kernel meal, investigating the simulated *in vitro* gastrointestinal digestion impact on defatted flour functional antioxidant and prebiotic potential source.

2. Materials and Methods

2.1. Samples

Semi defatted dehulled sunflower meal (SSM) and semi defatted palm kernel meal (SPM) were supplied by Veris Óleos Vegetais Ltda-ME, Vinhedo/SP (Brazil) and Agropalma, Moju/PA (Brazil), respectively. Enzymes were supplied by Sigma-Aldrich (α-amylase from porcine pancreas, pepsin from porcine gastric mucosa, pancreatin from porcine and porcine bile extract). Chemicals and enzymes were supplied by Sigma-Aldrich (α-amylase from porcine pancreas A3176, pepsin from porcine gastric mucosa P7000, pancreatin from porcine P1750, and porcine bile extract B8631, 2,2'-Azobis(2-amidinopropane)-dihydrochloride granular (AAPH), fluorescein sodium salt, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-difenil-1-picrilidrazil (DPPH), 6-hidroxi-

2,5,7,8-tetrametilcroman-2-carboxilic acid (Trolox), gallic acid), Thermo Scientific (SYBR safe), Dinâmica ($(NH_4)_2C_6H_6O_7$; $MnSO_4.H_2O$; K_2HPO_4;) KASVI (agar, yeast extract, beef extract, peptone bacteriological), Acumedia (peptic digest of animal tissue, peptone A), Synth ($NaC_2H_3O_2.3H_2O$; $MgSO_4.7H_20$, folin-ciocalteu reagent, n-hexane, ethanol, methanol, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, NaOH, HCl, $CaCl_2(H_2O)_2$), Neon (Polysorbate 80), Invitrogen (Ultrapure agarose). Na_2CO_3, 2,2,2-trichloroacetic acid (TCA), sodium phosphate buffer, TE buffer, TAE buffer.

2.2. Oilseeds defatted flour production and simulated *in vitro* digestion

SSM and SPM under took n-hexane extraction in Soxhlet equipment by 16 hours then, milled to 5 mm size, resulting in defatted sunflower flour (DSF) and defatted palm kernel flour (DPF). These material (5g) followed into *in vitro* gastrointestinal digestion according to Minekus et al. (2014). The DSF was added of deionized water (20 mL) and submitted to oral phase (pH 7, α-amylase, 10 min). Both gastric and intestinal phases were maintained for 2 hours duration, at pH 3 and 7 respectively. All phases ware conducted in water bath at 37 ºC under stirring (90 rpm). In order to stop the digestion, the whole volume was heated to 90 ºC by 10 minutes and cooled down to 4 ºC in cold water bath. In order to separate soluble (SDs) and insoluble (SDi) fractions, the digested material was centrifugated (3,645 x g; 30 minutes; 4 ºC) and freeze-dried. Aiming to increase the defatted palm kernel flour (DPF) digestibility through gastrointestinal enzymes, an autohydrolysis pretreatment was performed to promote hemicellulose solubilization (Sabiha-Hanim, Noor, & Rosma, 2011). An autoclave treatment was done (121 ºC, 15 minutes) in 20 mL of sodium acetate buffer 0.2 M, pH 5.4, (Cerveró, Skovgaard, Felby, Sørensen, & Jørgensen, 2010). Thereafter, the autoclaved defatted palm kernel flour (DPFa) was *in vitro* digested as describe above, excepted by no deionized water addition in oral phase. The autoclaved defatted palm kernel flour soluble (PDs) and insoluble (PDi) fractions were also freeze-dried.

2.3. Samples characterization

Physicochemical compositions: moisture, lipids, protein, ashes were performed according to AOAC methods (Latimer & George, 2012) and carbohydrate was determined by difference. Amino acid profile was carried out by pre-column derivatization of amino acids released after acid hydrolysis (6 mol/L), under heating (110º C/20 h), according to White, Hart and Fry (1986). The analysis of derivatized amino acids was carried out in a high-performance liquid chromatograph, HPLC (Shimadzu Corporation, Kyoto, Japan), with C18 column LUNA 100 Å (4.6 mm x 250 mm; particle size 5 μm) (Phenomenex, Torrance, CA, USA). Amino acids were quantified by comparison with amino acids standard Thermo Scientific (Rockford, IL, USA) and DL-2-aminobutyric acid (Sigma-Aldrich®, St.Louis, MO, USA) was used as an internal standard. The amino acid score was determined according to WHO/FAO/UNU (2007). Size Exclusion Fast Protein Liquid Chromatography, SE-FPLC, (AKTA Pure 25, GE Healthcare, Superdex-200 10/300 GL and Superdex-30 10/300 GL columns and software Unicorn 6.3) was used to obtain the molecular weight (MW) distribution profile according to the aromatic compounds retention time (Vander Heyden, Popovici, & Schoenmakers, 2002). Samples were solubilized (5 mg / mL) in sodium phosphate buffer (25 mM pH 7,4 with 150 mM NaCl), sonicated for 10 minutes and filtered in 45 μm polytetrafluoroethane membrane before injection (500 μL). The isocratic elution in flow 0,5 mL / minutes was performed at Akta Pure chromatography (GE Healthcare, Chicago, Illinois, EUA) with Superdex 200 in series with Superdex 30 column during 90 minutes and UV-Vis 280 nm detector. The standards α-lactalbumin (14,178 Da), Insulin (5,807.6 Da), Vitamin B12 (1,355.37 Da) and L-β-4-dihydroxyphenylanine (197.2 Da) were used to build an analytical curve, from which it was possible to establish MW ranges.

2.4. Extract preparation

Phenolic compounds and antioxidant capacity by ORAC, ABTS, DPPH and DNA protective capacity assays were determined based on extract preparation, carried out according to Kim, Jeong and Lee (2003), with some modifications. Trying to investigate bioactive compounds solubility, three diferentes fluids (deionized water, aqueous ethanolic solution (70:30 v/v) and aqueous methanolic solution (70:30 v/v)) were used as extractor solvents. Samples (100 mg) of DSF, DSF soluble fraction ($DSF_{soluble}$), DPF, DPFa and DPFa soluble fraction ($DPFa_{soluble}$), were solubilized into 4 mL of solvent and took under one-minute stirring in Ultra-Turrax T-25, followed by centrifugation (1125 x g, 4 ^{o}C, 10 minutes). The soluble phase was filtrated in Whatman paper n°2 into a 10 mL volumetric flask. This procedure was repeated twice and the volume (10 mL) was completed with solvent. Each extract was split in several eppendorfs which were kept at -10º C. These main extracts were tested in the assays cited above, adjusting the extract dilution, if necessary.

2.4.1. Total phenolic compounds

Total phenolic compounds was determined by Folin-Ciocalteau reagent (Al-Duais, Müller, Böhm, & Jetschke (2009). Briefly, 100 µL of Folin-Ciocalteau 10% was mixture with 20 µL of sample extract (aqueous, ethanolic or methanolic extracts as describe above). After 5 minutes, the reaction mixture was added 75 µL of 7,5% (w/v) Na_2CO_3 and maintained in darkness for 40 minutes reaction after reading at 740 nm wavelength UV-Vis microplate spectrophotometer Synergy (BioTek®, with Gen5 software). The same procedure was done with deionized water in order to discount the blank absorbance. A gallic acid standard curve solution was used to express the results in mg GAE / g sample.

2.4.2. Oxygen radical absorbance capacity

Oxygen radical absorbance capacity (ORAC) assay was performed as preview describe by Chisté et al., 2011, using 2,2'-Azobis(2-amidinopropane)-dihydrochloride granular (AAPH) as reactive oxygen species (ROS) generator. Therefore, it measured the extract antioxidant capacity to protect fluorescein probe from peroxyl radical oxidation. The assay was carried out on fluorescence 96-wells microplate Synergy reader (BioTek®, software Gen5) with fluorescence filters for excitation and emission wavelength of 493 nm (485/20) and 515 nm (528/20), respectively. Stock fluorescein sodium salt solution (4,066 mM) was prepared by 15.3 mg fluorescein dissolved in 10 mL phosphate buffer (PSB, 75 mM, pH 7.4). The work solution was obtained by two step dilution: 100 µL of stock solution into 10mL PSB followed by 125 µL of this intermediate solution into 10 mL of PSB. Reaction mixture in sample well contained antioxidant extract (30 µL) and fluorescein work solution (60 µL) was preincubated at 37°C for 15 minutes followed by 110 µL AAPH solution (76 mM prepared in PSB) addition and incubation for two hours. The samples and standard net fluorescence decay (AUC_{net}) was calculated by the difference between the area under the fluorescence decay curve of samples/standard ($AUC_{sample/standard}$) and 200 µL PSB (AUC_{blank}). A 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxilic acid (trolox) standard curve was used to expresses the results as µmol Trolox equivalent/g sample.

2.4.3. ABTS radical scavenging assay

ABTS radical scavenging capacity was measured according to Al-Duais, Müller, Böhm and Jetschke, 2009. Radical $ABTS^{•+}$ was generated by the addition of 88µL of aqueous persulfate potassium $K_2S_2O_8$ (140 mM) into 5 mL of 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) aqueous solution (7mM). After 16 hours remaining in darkness, the ABTS working solution had its absorbance adjusted to 0.7±0.02 at 734 nm in UV-Vis spectrophotometer. Reaction mixture occurred into 96-well microplate, adding 20µL of samples extract and 220µL of ABTS working solution. The absorbance was

read at 730nm in UV-Vis microplate Synergy reader (BioTek®, software Gen5) after six minutes. Blank was composed of 240 μL of phosphate buffer (PSB; 75mM; pH 7.4). Trolox standard curve was prepared in PSB and results were expressed as μmol Trolox equivalent/g sample.

2.4.4. DPPH radical scavenging assay

2,2-Diphenyil-picrylhydrazyl (DPPH) radical scavenging capacity was performed in 96-well UV-Vis microplate Synergy reader (BioTek®, software Gen5) according to Al-Duais, Müller, Böhm, & Jetschke, 2009. In each sample well 134 μL of ethanolic DPPH solution (150μM) was added to 66 μL of extract. Reaction mixture was kept in darkness for 45 minutes before read at 517 nm. The control well has 66 μL of ethanol instead sample and the blank, just 200 μL of ethanol. A trolox standard curve in ethanol was made and the antioxidant capacity was calculated as μmol trolox equivalent/g sample.

2.4.5. DNA supercoiled band protective capacity

Plasmidial DNA pcDNA-FLAG (Pavan et al., 2016) was used in a 0.8% agarose gel electrophoresis to evaluate DSF, DPF, SDs and PDs aqueous extract antioxidant capacity against peroxyl radical, generated by AAPH thermal degradation (Yarnpakdee, Benjakul, Kristinsson, & Bakken, 2015). In order to eliminate any intrinsic proteolytic enzyme, the DSF and DPF was heated at 90° C by 10 minutes. In a DNAse free microtube, the following reagents were added at this stated order: 4 μL of DNA plasmidial supercoiled pcDNA (125 ng / mL) prepared at Tris-EDTA buffer, 2 μL of samples extract, 4 μL of 30 mM aqueous APPH solution. In order to have a DNA supercoil band intensity control for native DNA form (positive control), 6 μL of deionized water were added instead sample and AAPH. Also, a DNA supercoil band intensity control for oxidized DNA form (negative control) was preformed, by adding 2 μL of deionized water instead the sample volume. All these treatments were incubated in darkness at 37° C for 1 hour. The reaction mixture (10 μL) was

applied in a 0.8 % agarose gel electrophoresis using Tris-acetic acid ETDA buffer, running at 80 mV for 1.5 hours followed by 120 mV during 1 hour. The gel was stained with 1:20.000 SYBR safe (Thermo Scientific) added to TAE-buffer and then the bands were visualized under UV light at ChemiDoc Imaging System (Bio-Rad). The quantification was done in Image J software. The results were expressed as following equation (2).

$$Retention\ supercoiled\ DNA\ band\ (\%) = \frac{intensit\ of\ sample\ supercoiled\ band}{intensit\ of\ control\ supercoiled\ band} * 100 \quad \text{(eq. 2)}$$

2.5. Prebiotic effect

To prospect a potential prebiotic effect of SDi and PDi, probiotic commercial cultures of *Lactobacillus plantarum* BG112 (recently renamed as *Lactiplantibacillus plantarum* BG112 according to Zheng et al. (2020)), *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* BLC1 and *Bifidobacterium lactis* BB-12 were inoculated at MRS medium formulated from its individual components, in order to control the carbohydrate source (Moreno-Vilet et al., 2014). The standard positive MRS control medium (MRS_{pc}) was formulated by the following compounds solubilized in 200 mL of deionized water: sodium acetate (1g), agar (3g), dibasic ammonium citrate (0.4g), peptic digest of animal tissue (peptone A, 2g), beef extract (2g), yeast extract (1g), potassium phosphate (0.4g), magnesium sulfate (0.02g), manganese sulfate (0.01g), 200 µL of polysorbate 80 and dextrose (4g). In order to have MRS media with no carbon source addition, a negative MRS control medium (MRS_{nc}) formulation was done and it had no dextrose addition. Through Di physicochemical composition, S-Di showed a lower carbohydrate content than P-Di, so it was used as a parameter to replace dextrose in the plates that carried Di samples. The dextrose (4g) was substituted by SDi (4g, MRS_{SDi}), which had 1.89 g of carbohydrates. Based on this amount of carbohydrate, 2.14g of PDi was added instead dextrose and made the MRS_{PDi}. Two more growth control media were done, first through dextrose replacement by inulin (1.89 g inulin MRSinulin) and second, by the reduction of dextrose content (adjusted positive control

MRSapc, 1.89 g dextrose) All the five MRS mediums were autoclaved. Freeze-dried commercial probiotic serial dilution was resuspended in peptone water 0.1% (w/v) and spread plate technique was used for plate counting. *Lactobacillus* incubation was carried out in microaerophilia and *Bifidobacterium* in anaerobiosis (Anaerogen), both for 72 hours at 37 ºC. After the incubation time, the colonies forming units were counted and the result was expressed as log cfu / g. All probiotics and medium where compared to the MRS_{nc}.

2.6. Statistical analysis

The results were expressed as means ± SD (standard deviation) of three measurements. For all assays were applied one-way ANOVA with Tuckey test, at significance level of $p<0.05$, excepted for DNA protective capacity and potential prebiotic effect assay, that was applied one-way ANOVA with Dunnet test (also at significance level of $p<0.05$). The statistical program was GraphPad Prism 8.0.1.

3. Results and Discussion

3.1. Samples characterization

Defatted sunflower flour (DSF), defatted sunflower flour digested soluble (SDs) and insoluble (SDi) fractions, besides defatted palm kernel flour (DPF), autoclaved defatted palm kernel digested soluble (PDs) and insoluble (PDi) fractions were physicochemical characterized as showed in Table 1. Regarding to the macro components, DSF is a better source of protein than DPF, which is enriched in carbohydrates. After GID, 0.36 ± 0.01g (7.94 ± 0.12%) of DPF become soluble. The amount of protein in P-Ds and P-Di were 0.09 ± 0.01 g (24.07 ± 0.38 %) and 0.27 ± 0.01 g, respectively. On the other hand, 1.95 ± 0.06 g (42.72 ± 1.34%) of DSF become soluble after GID, that contains 1.24 ± 0.04 g of protein (51.91 ± 1.63 %). The carbohydrates were the largest SDi and PDi components, suggesting that they were poorly digested by gastrointestinal enzymes.

Table 1. Physicochemical composition of sunflower and palm kernel' samples in dry matter.

Components (%)	DF	Ds	Di
Sunflower' samples (S)			
Lipids	0,37 ± 0,01	nd	nd
Protein	52,41 ± 0,02 b	63,68 ± 0,06 a	36,76 ± 0,17 c
Ashes	8,76 ± 0,05 c	15,04 ± 0,09 b	15,28 ± 0,06 a
Carbohydrates	38,47*	21,28*	47,96*
Total dietary fibers	18.00 ± 0.39		32.63 ± 0.18
Palm Kernel' samples (P)			
Lipids	0.37 ± 0.00	nd	nd
Protein	7.82 ± 0.13 b	23.69 ± 0.01 a	7.37 ± 0.05 c
Ashes	2.84 ± 0.02 c	44.34 ± 0.07 a	2.97 ± 0.00 b
Carbohydrates	88.97*	31.97*	89.67*
Total dietary fibers	85.54 ± 0.11		85.46 ± 0.09

Values are means ± standard deviations of three measurements. Values with different superscript letters in the same row are significantly different at $p<0.05$. Abbreviation includes DF: defatted flour; Ds: digested soluble fraction; Di: digested insoluble fraction. (*): Carbohydrate was determinate by difference. nd: not determined.

The sunflower proteins are composed mainly by globulins (40% - 90%) and albumins (10% - 30%), and the 11 S globulin and the 2 S albumin the most relevant fractions (Pickardt, Eisner, Kammerer, & Carle, 2015). The indispensable amino acids (except by lysine) attempt to FAO standard requirements, configurating the sunflower flour a high nutritional quality protein source (Alexandrino, Ferrari, de Oliveira, de Cássia S.C. Ormenese, & Pacheco, 2017). Sunflower protein hydrolysate by pepsin and pancreatin has generate peptides that showed Cooper-chelating activity (Sarmadi & Ismail, 2010).

The palm kernel carbohydrates are major mannoses (78%), cellulose (12%), glucuronoxylans and arabinoxylans (6%) linked by β1→4 glycosidic band, which make them resistant to human gastrointestinal enzymes (Bello et al., 2018). Regarding to palm kernel protein, study has isolated two peptides (amino acid sequences GIFE e LPWRPATNVF) with antioxidant activity by DPPH assay and metal-binding capacity (Zarei et al., 2014) and antimicrobial peptides from Alcalase® hydrolysis (Tan, Ayob, & Wan Yaacob, 2013). Therefore, palm kernel might be a source of antioxidant peptides.

The MW distribution profile from the two matrixes are different and help to clarify the GID impact on the flours. At the analysis condition, SFD has more soluble compounds than DPF. After SDF and DPF digestion, the total area increased, with a large amount of high MW compounds in PDs and smaller compounds in SDs. SDs and PDs molecular weight (MW) distribution profile showed that, at the analysis condition, soluble compounds which had MW<3kDa represented 73.47% and 35.06% of SDs and PDs samples. The SDs and PDs area under the curve was higher than the DSF and DPF (Figure 1). That revels the effectiveness of gastrointestinal enzymes on digesting and solubilizing flour compounds. The P-Ds had a high intense pick around 66 kDa to 44 kDa while S-Ds most compounds showed MW lower than 1.340 Da.

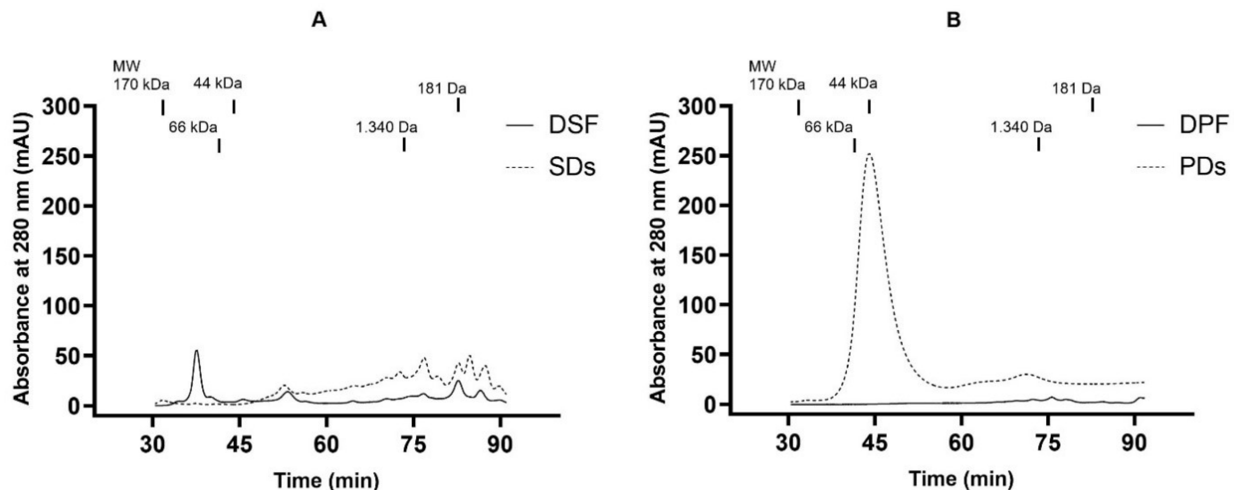

Figure 1. Molecular weight distribution by FPLC-SE. (A) Sunflower' samples: defatted sunflower flour, DSF (continuous line) and defatted sunflower flour digested soluble fraction, SDs (dotted line) (B) Palm kernel' samples: defatted palm kernel flour, DPF (continuous line) and autoclaved defatted palm kernel digested soluble fraction, PDs (dotted line).

According to WHO/FAO/UNU (2007) DSF and DPF have the most indispensable amino acid being important protein source. The limiting amino acid in DSF was lysine and at DPF, they were lysine and sulfur amino acids (met+cys). The GID has increased some amino acids concentration in SDs (Trp, Thr, Asp, Glu, Ser, Arg, Pro, Gly) and in PDs (Lys, Trp, Met+Cys, Thr, His, Asp, Glu, Ser, Pro, Gly) as shown in Table 2.

Table 2. Sunflower and palm kernel samples amino acid score.

Amino acids (AA)		WHO/FAO/UNU mg/g ref.protein	DSF		S-Ds		DPF		P-Ds	
			mg/g protein	Score	mg/g protein	Score	mg/g protein	Score	mg/g protein	Score
Indispensable	Lys	45	38.83 ± 0.04	0.86	36.91 ± 0.02	0.82	28.56 ± 0.01	0.63	36.07 ± 0.21	0.80
	Trp	6	nd	0.00	6.37 ± 0.00	1.06	0	0.00	6.74 ± 0.18	1.12
	Phe + Tyr	38	74.36 ± 0.08	1.96	74.22 ± 0.09	1.95	80.51 ± 0.08	2.12	64.83 ± 0.33	1.71
	Met + Cys	22	38.19 ± 0.02	1.74	29.61 ± 0.07	1.35	19.87 ± 0.01	0.90	31.80 ± 0.33	1.41
	Thr	23	35.93 ± 0.04	1.56	37.71 ± 0.03	1.64	34.90 ± 0.02	1.52	37.85 ± 0.37	1.65
	Leu	59	64.24 ± 0.55	1.09	59.32 ± 0.03	1.01	74.76 ± 0.01	1.27	56.32 ± 0.49	0.95
	Ile	30	45.56± 0.48	1.52	41.43 ± 0.06	1.38	38.92 ± 0.07	1.30	38.41 ± 0.33	1.28
	Val	39	58.22 ± 0.05	1.49	50.16 ± 0.22	1.29	56.86 ± 0.16	1.46	51.09 ± 0.33	1.31
	His	15	27.12 ± 0.14	1.81	26.16 ± 0.03	1.74	15.91 ± 0.01	1.06	17.62 ± 0.18	1.17
Dispensable	Asp		106.66± 0.14	UN	111.15 ± 0.07	UN	87.11 ± 0.52	UN	112.79 ± 0.78	UN
	Glu		220.45± 0.28	UN	228.77 ± 0.18	UN	194.86 ± 0.03	UN	198.01 ± 0.02	UN
	Ser		46.28± 0.15	UN	48.15 ± 0.05	UN	54.50 ± 0.01	UN	55.35 ± 0.49	UN
	Arg		90.81 ± 0.23	UN	92.72 ± 0.03	UN	149.74 ± 0.27	UN	121.40 ± 1.09	UN
	Ala		44.48 ± 0.12	UN	43.17 ± 0.13	UN	52.71 ± 0.03	UN	45.11 ± 0.40	UN
	Pro		38.32 ± 0.12	UN	45.27 ± 0.12	UN	46.16 ± 0.08	UN	47.79 ± 0.52	UN
	Gly		57.94 ± 0.28	UN	68.88 ± 0.16	UN	64.63 ± 0.04	UN	79.51 ± 0.71	UN
AA distribution	Hydrophobic		32,70 %		31,55 %		33,49 %		29,55 %	
	Hydrophilic		48,39 %		49,57 %		47,62 %		48,59 %	
	Neutral		18,91 %		18,88 %		18,89 %		21,86 %	

Values are means ± standard deviations of three measurements. Protein determinate by Kjeldahl. Abbreviations includes: AA: amino acid; UN: unvalued; nd: not detected; Asp: Aspartic acid; Ala: Alanine; Arg: Arginine; Gln: Glutamine; Gly: Glycine; His: Histidine; Cys: Cysteine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Glu: glutamic acid; Tyr: Tyrosine; Val: Valine. Hydrophobic (Ala, Val, Met, Phe, Leu, Ile, Pro, Trp), Hyrophilic (Arg, Asp, His, Lys, Glu) and Neutral AA (Ser, Gly, Thr, Tyr, Cys). Score to adults > 18 years (FAO, 2007).

3.2. Antioxidant assays and phenolic compounds content

The results for phenolic compounds, ORAC, ABTS and DPPH assays are shown at Table 3. The DSF and SDs have shown higher phenolic compounds content than DPK, DPKa and PDs and it might be correlated to higher antioxidant capacity by ORAC, ABTS and DPPH methods, independent of the solvent used.

Without a doubt, phenolic compounds have been described as radical quenching by either electron transfer or proton donation (Silva et al., 2017). Antioxidant capacity has also been attributed to some peptides and soluble dietary fibers (Mrabet et al., 2017; Sarmadi & Ismail, 2010). Sunflower and palm kernel samples showed higher antioxidant capacity through ORAC method. The peroxyl radical (ROO•) was produced by AAPH's thermo decomposition (Zulueta, Esteve, & Frígola, 2009). Leonard et al. (2006) suggested that ROS damage on DNA molecule could be affect by scavenging of radicals formed during reaction or by inhibiting the radical generation. Dietary antioxidant may help to maintain the body redox homeostasis, avoiding oxidative stress and cellular macromolecule damage, which is connect to mutagenesis and chronic disease development (Zhang, Mu, & Sun, 2014).

For sunflower' samples, the highest antioxidant response was shown by methanolic and ethanolic extracts in ORAC assay, which were more effective to solubilize DSF's antioxidants compounds than water. By DPPH and ABTS mechanism, the DSF compounds showed similar antioxidant activity and the three extracts didn't show difference regarding to phenolic compounds extraction efficiency. The GID were successful process to release compound with antioxidant capacity, as phenolic compounds. The major phenolic acid in sunflower kernels is chlorogenic acid, with low caffeic acid (Alu'datt et al., 2017). The highest antioxidant

capacity was showed at ethanolic extract in ORAC assay (1009.6 ± 6.05 µmol Trolox eq. /g sample) and there was no difference among the solvents to phenolic compounds extraction. Thus, hydroethanolic solvent (30:70 v/v) might be a good phenolic and antioxidant sunflower compounds solvent.

For palm kernel' samples, they showed lower antioxidant capacity and phenolic content than sunflower samples. However, it also showed higher antioxidant capacity by ORAC assay. Regarding palm kernel flour (with and without autoclave treatment), methanolic and ethanolic extracts were better solvents than water to extract antioxidant components from the defatted flour (DF). The autoclave did not represent any difference to increase the overall DF and DFa's antioxidant capacity. After the GID, the best antioxidant capacity was produced by aqueous extract in ORAC assay (314.1 ± 16.45 µmol Trolox eq. /g sample) with no difference to ethanolic extract (297.9 ± 6.16 µmol Trolox eq. /g sample). The PDs had higher antioxidant activity by ABTS than DPPH radical scavenging activity, with no difference between solvents. In general, GID had a positive impact on releasing antioxidant compounds and phenolics in both by-product matrixes.

Table 3. Antioxidant capacity by ORAC, ABTS and DPPH methods besides total phenolic compounds in different solvent extraction.

Assay (µmol Trolox eq. /g sample)	Extract	DSF	SDs	DPF	DPFa	PDs
ORAC	Aqueous	642.3 ± 43.79 b,A	678.7 ± 1.26 c,A	6.6 ± 1.58 c,C	8.8 ± 0.63 c, C	314.1 ± 16.45 a, B
	Ethanolic	729.8 ± 42.42 a,B	1009.6 ± 6.05 a,A	40.5 ± 1.10 a,D	87.0 ± 1.69 a, D	297.9 ± 6.16 a, C
	Methanolic	758.5 ± 42.42 a,B	923.3 ± 28.01 b,A	41.5 ± 3.43 a,D	84.1 ± 5.63 a, D	280.8 ± 4.03 b, C
ABTS	Aqueous	117.41 ± 1.34 d,B	191.95 ± 0.60 e,A	1.82 ± 0.02 d,D	1.88 ± 0.01 c, D	66.75 ± 1.74 c, C
	Ethanolic	142.28 ± 8.19 cd,B	174.77 ± 3.94 e,A	13.23 ± 0.63 b,D	23.30 ± 0.61 b, D	80.52 ± 7.52 c, C
	Methanolic	156.90 ± 5.06 cd,B	187.37 ± 4.55 e,A	12.04 ± 0.50 b,D	18.30 ± 0.43 b, D	73.22 ± 1.60 c, C
DPPH	Aqueous	157.70 ± 10.57 d,B	252.09 ± 5.05 d,A	1.19 ± 0.01 d,C	1.15 ± 0.03 d, C	5.50 ± 1.20 d, C
	Ethanolic	173.25 ± 5.75 cd,B	240.13 ± 5.75 d,A	6.19 ± 0.18 c,C	6.35 ± 0.05 cd, C	2.53 ± 0.18 d, C
	Methanolic	194.91 ± 11.16 c,B	264.28 ± 2.19 d,A	6.02 ± 0.17 c,C	6.29 ± 0.03 cd, C	4.83 ± 1.21 d, C
Assay (mg Gallic Acid /g sample)	**Extract**	**DSF**	**SDs**	**DPF**	**DPFa**	**PDs**
Phenolic Compounds	Aqueous	20.10 ± 1.14 e,B	29.23 ± 0.89 f,A	0.27 ± 0.01 d,D	0.31 ± 0.01 d,D	9.14 ± 0.48 d,C
	Ethanolic	18.28 ± 0.27 e,B	24.87 ± 0.85 f,A	1.33 ± 0.00 d,D	2.20 ± 0.06 cd,D	8.52 ± 0.22 d,C
	Methanolic	18.06 ± 0.42 e,B	25.07 ± 0.09 f,A	1.12 ± 0.04 d,E	1.84 ± 0.00 d,D	7.76 ± 0.09 d,C

Values are means ± standard deviations of three (3) measurements. Different small letters in the same column and different capital letters in the same row indicate difference at significant level $p \leq 0.05$. Abbreviations includes: DSF = defatted sunflower flour; SDs = sunflower digested soluble fraction; DPF = defatted palm kernel flour; DPFa = defatted palm kernel flour after autoclave; PDs = palm kernel digested soluble fraction.

Regarding to the DNA supercoil protective capacity, the Figure 2 shows the agarose electrophoresis gel (2a) and the results after analysis on software ImageJ (2b). The SDs showed an increase in 3.45% on the protective capacity compared to the DSF, but both were different from the DNA positive control. Therefore, SDs and DSF didn't protected the DNA from APPH oxidation. On the other hand, the GID increased in 2.22 folds the DPF after autoclave antioxidant capacity becoming able to safeguard the DNA molecule (92.65% of DNA supercoil band was maintained).

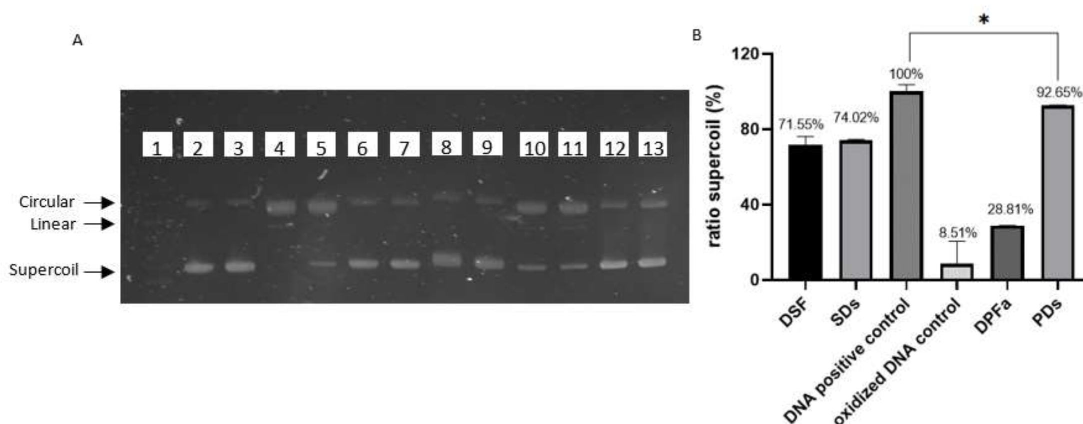

Figure 2 DNA Protection assay. Figure (3a) the agarose gel electrophoresis which numbers refer to *1: molecular weight standard; 2 and 3: DNA positive control; 4 and 5: oxidized DNA control (DNA+AAPH); 6 and 7: DNA+AAPH+ deffated sunflower flour (DSF); 8 and 9: DNA+AAPH+ deffated sunflower gastrointestinal digested soluble fraction (SDs); 10 and 11: DNA+AAPH+ deffated palm kernel autoclaved flour (DPFa); 12 and 13: DNA+AAPH+ deffated palm kernel autoclaved gastrointestinal digested soluble fraction (PDs). Figure 3b. relationship between DNA positive control and other treatments. Samples with (*) have no difference to DNA positive control.*

The antioxidant activity by ORAC and DNA protection capacity had the same pro oxidant radical, the peroxyl, produced by AAPH thermo degradation. Although SDs showed higher antioxidant capacity and total phenolic compounds in aqueous extract than PDs, just PDs could develop a DNA protective activity, probably related to a different compound to phenolic compounds. Further analysis should be conducted aiming to define the pathway used by PDs compound.

Both antioxidant capacity and phenolic content in SDs and PDs samples were higher than the original flour, showing a GID positive impact on releasing compounds prior entrapped in food matrixes, so becoming more bioaccessible. It may be an indication of potential biofunctionality to foods which have DSF and DPF in their composition. Antioxidant *in vitro* assays (antioxidant capacity and phenolic compounds) are useful tools to investigate potential dietary antioxidant sources as a screening method with low cost. Although antioxidant activity depends on complex interactions between food matrix and organism that cannot be predict by simple chemical reactions (Granato et al., 2018). Therefore, next research steps might be the application of these flours in real food products, the isolations and identification of the specific compounds besides the usage of cell model trying to predict mechanism of action.

3.3. Potential prebiotic effect

Different control mediums were used to investigate the potential prebiotic effect of SDi and PDi. The difference among these samples, as the only carbon source, didn't present any difference to negative control (MRSnc), as showed in the Figure 3. Unlike to Moreno-Vilet et al. (2014), the negative control had a bacterial growth superior than 8 log cfu/g and it has impacted on the comparison to other media. However, some studies have successfully isolated prebiotic compounds from palm kernel matter.

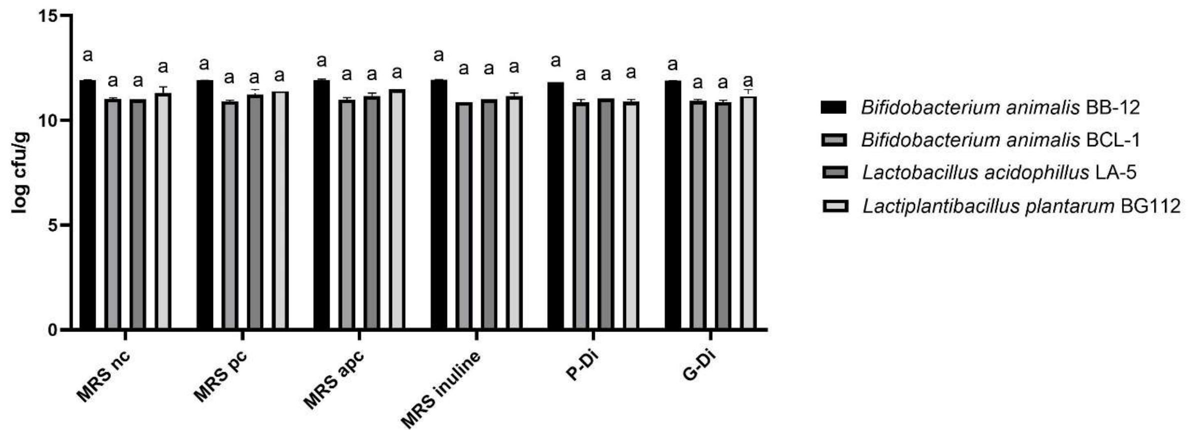

Figure 3 Prebiotic assay .Abbreviations include: defatted palm kernel flour digested insoluble fraction (PDi) added in MRS media as the only carbon source (P-Di), defatted sunflower flour digested insoluble fraction added in MRS media as the only carbon source (S-Di), negative control (MRSnc): didn't have any carbon source added. Standard positive control (MRSpc) and adjusted positive control (MRSapc) had dextrose addition and prebiotic control (MRSinulin), had inulin added.

It has been reported that the intestinal microflora ferment shorter oligosaccharide chains more effectively compared to the larger oligosaccharide chains (Kalidas et al., 2017). Few had been discussed about sunflower prebiotic effects. The definition of host and target side need to be specified. For example cellulose can be considered a prebiotic in ruminants but not in humans, as the latter's intestinal microbiota only poorly utilize β(1→4) linked d-glucose polysaccharides (Gibson et al., 2017).

Recent studies have describe prebiotic effect of mannanoligosaccharides extract from palm kernel cake with *Lactobacillus reuteri* C1 proliferative effect (Kalidas et al., 2017) and also with *Lactobacillus plantarum* ATCC8014 and *Lactobacillus rhamnosus* ATCC 53103 (Bello et al., 2018). These microorganisms were recently renamed as *Limosilactobacillus reuteri*, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* (ZHENG et al., 2020).

4. Conclusion

The valorization, management and reuse of edible oil industry by-products is an important aspect leading to sustainable development. Sunflower and palm kernel meal are source of bioactive compounds, such proteins, phenolic compounds and polysaccharides. The GID of defatted sunflower flour was able to release compounds with antioxidant capacity and phenolic compounds. The hydroethanolic solution (30:70 v/v) was the best solvent in order to match both goals. The SDi and PDi were not able to stimulate the proliferations of any probiotic bacteria tested mainly by the large growth in the MRSnc. Regarding to the high carbohydrates content in DPF, the autoclave process associated to GID were able to release compounds poorly antioxidant by ORAC, ABTS and DPPH assay however with DNA protection capacity. Further studies should be done to investigate biodisponibility in Caco-2 cell model aiming to confirm antioxidant capacity. It was not observed a potential probiotic effect from sunflower and palm kernel meal. Further studies may improve human food security by incorporation of oil industry by-products.

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CONCLUSÃO GERAL

A demanda por alimentos que promovam a saúde e o bem estar dos consumidores continua em ascensão. Ingredientes que de diversas formas possam reduzir o risco de doenças crônico degenerativas não-transmissíveis são valorizadas por consumidores e nesse cenário, destacam-se os antioxidantes, os prebióticos e probióticos. Dentro do contexto de sustentabilidade, no âmbito de economia circular e de valorização de co-produtos agroindustriais, as farinhas de oleaginosas constituem uma fonte de compostos bioativos valorosos, como proteínas, compostos fenólicos e prebióticos (como polissacarídeos não amiláceos). A digestão gastrointestinal simulada (DGI) das farinhas desengorduradas de castanha-de-caju, girassol e de palmiste pré-tratada mostraram melhora da bioacessibilidade de proteína e compostos fenólicos, percebida pelo aumento de compostos solúveis no digerido (Ds), aumento da concentração de compostos de peso molecular < 3 kDa. A DGI também promoveu melhora da atividade antioxidante em todos os solventes extratores testados (água destilada, etanol 70% e metanol 70%) nos métodos aplicados (ABTS, DPPH, ORAC e proteção ao DNA). A fração insolúvel do digerido (Di), a qual representa a porção não digerida pelas enzimas digestivas e fermentescível pelas bactérias do cólon, estimulou à proliferação de *Bifidobacterium lactis BB-12* em meio MRS, enquanto a mesma fração das demais farinhas não apresentou diferença com o controle. Muitos outros requisitos precisam ser estudados para classificar o material como prebiótico ou capaz de gerar um efeito prebiótico, sendo necessário ampliar o número de testes e avaliar outros aspectos além do estímulo de crescimento das bactérias ácido láticas. Diferenças na composição das farinhas analisadas levam a trabalhar abordagens de aplicação individualizadas para cada uma delas. A farinha de castanha-de-caju poderia ser empregada para fortificação de alimentos, a de girassol poderia ser explorada em separado as frações proteicas e de polifenóis, enquanto a de palmiste poderia ser destinada a produção de prebióticos.