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

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ABSTRACT

Cashew nuts are mainly consumed as a roasted and salted snack. Lately, the industry has gained interest in broken kernels because of their added value. In this study, defatted cashew nut flour (DCF) underwent simulated gastrointestinal digestion to obtain a soluble (CDs) and an insoluble (CDi) digested fraction. These fractions, which resulted from the digestion of a complex matrix, were evaluated for antioxidant capacity of bioaccessible compounds (present on the soluble digested fraction, CDs) and their potential prebiotic effect, considering that the insoluble digested fraction (CDi) could be fermented by the microbiota in the gut. The DCF had a high protein content (40.74%), being nutritionally characterized as a balanced source of amino acids, with a predominance of aromatic amino acids (phenylalanine and tyrosine), threonine and histidine. The digested DCF presented 76.90% of the soluble components of low molecular weight (0.1–2 kDa), which is typical of antioxidant peptides. The soluble digested fraction (CDs) significantly increased the antioxidant capacity in relation to flour in the ORAC and ABTS assays and the aqueous extract presented the highest values (526.0 and 76.64 as μmol Trolox Eq./g sample, respectively). The CDs protected 29.03% of the supercoiled DNA band and ratified the potential antioxidant capacity after GID in a physiological assay. In addition, the insoluble digested fraction showed a potential prebiotic effect for *Bifdobacterium lactis* BB-12. Finally, simulated gastrointestinal digestion improves the bioaccessibility of CDF antioxidant compounds as a complex matrix, containing low molecular weight peptides and phenolic compounds, which become more available to react with reactive oxygen species (ROS). In addition, the potential prebiotic effect of defatted cashew nut flour has yielded a promising solution for the total reuse of broken cashew nut kernel as a functional food ingredient.

1. Introduction

Initiatives have been undertaken globally to address food security, human health, sustainable development and environmental preservation, and these are driven by the concept of a circular economy (EU, 2020). The reuse of by-products generated by agribusiness through the extraction and conversion of biocomponents, into more valuable products, constitutes a global trend to ensure the development of a more competitive and sustainable enterprise. Within this scenario, several matrices for different food production processes have been widely studied (Contreras et al., 2019).

Anacardium occidentale L. is a native Brazilian tree and the most widely known specie of the Anacardiaceae family, due to its cashew nut's nutritional and socio-economic value (Oliveira, Mothé, Mothé, &

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de Oliveira, 2020; Shahidi & Zhong, 2015). Cashew nut is among the most produced tree nut globally, mostly in Africa, India, Vietnam, Cambodia, Brazil, and Indonesia. The total production was of 7.86 million tons in the 2019/20 harvest (INC, 2020). According to the National Supply Company (CONAB, 2020), in 2019, Brazilian production of whole cashew nut within the shell was of 139.38 million tons in a total planted area of 425,797 ha.

The drivers identified in the market include the growing demand for healthy snacks, an increasing consumer market from Europe, and an increase in the number of primary processing units (Report Linker, 2020). Industrial processing of cashew nuts may generate about 40% of broken kernels, whose commercial value is much lower than the whole nut (de Carvalho, de Figueiredo, de Sousa, de Luna, & Maia, 2018). There are reports that roughly 30% of total cashew kernels are unacceptable for sale and disposed of because of bruises, damage, oiliness or scorching during drying. As cashew nut kernel profitability depends on kernel extraction without breaking or damaging, other by-products such as flour, oil, and butter have been studied as a more profitable use of broken kernels (Oliveira et al., 2020), for food product application (Sharma et al., 2020).

Cashew kernels are used in the preparation of a variety of food products such as cakes, sweets, ice cream, biscuits and chocolates for their characteristically pleasant taste (Chandrasekara & Shahidi, 2011). The whole kernels contains oleic (C18:1, 57.27%) and linoleic (C18:2, 20.80%) acids, plant proteins, selenium, phytosterols, tocopherols, high starchy content, crude fiber, and alkyl-phenols, which are industrially and nutritionally relevant (Sharma et al., 2020). Previous research has shown that the cashew nut oil's oxidative stability and phenolic compounds imply health benefit to humans and the broken kernel size does not interfere on the oil production (de Carvalho et al., 2018). Defatted cashew flour has been recommended as a nutritious protein source with potential applications due to its higher protein content and good balance of indispensables amino acids. Despite the increasing demand for plant proteins, more detailed functional and molecular data are required to better understand the potential advantages of the cashew nut (Liu et al., 2018).

Antioxidant compounds have been extensively studied against oxidative stress and for preventing or delaying several non-transmissible chronic diseases such as diabetes mellitus, various forms of cancer, neurodegenerative and coronary heart disease (Rusu, Gheldiu, Mocan, Vlase, & Popa, 2018). Phytochemicals and antioxidant peptides can be founded in plant by-products (Görgüç, Gençdağ, & Yılmaz, 2020; Lucini Mas et al., 2020). Regardless of their antioxidant capacity, nuts and edible seed compounds have been described as able to improve the microbiota in the gut (Sugizaki & Naves, 2018) since phenolics, oligosaccharides, and polyunsaturated fat acids (PUFA) are prebiotics and may develop a prebiotic effect, which means the promotion of "a selectively stimulate bifidobacteria, lactobacilli or other species beyond these genus, evoking a measurable net benefit to the host's health, distinct from a control" (Gibson et al., 2017).

In vitro digestion is a widely employed method to predict the gastrointestinal behavior of a food product. It represents a lower cost, is faster, is more resource efficient and has no ethical restrictions compared to human trials (Minekus et al., 2014). Furthermore, *in vitro* gastrointestinal digestion has been used to predict bioaccessibility of food compounds (Cilla, Bosch, Barberá, & Alegría, 2018). Some studies have shown the importance and highlighted the value of defatted meal from other nuts and seeds, such as Brazil nut, macadamia, sapucaia nut, amaranth and chia (Lucini Mas et al., 2020; Navarro & Rodrigues, 2016; Rodríguez & Tironi, 2020; Santos et al., 2013; Teixeira, Ávila, Hornung, Barbi, & Ribani, 2018)

In this context, the present research group was unable to find a single study regarding defatted cashew nut kernel flour's antioxidant and potential prebiotic within the literature. Therefore, the main objective of this research is to evaluate the impact of *in vitro* gastrointestinal digestion on defatted cashew nut kernel flour compounds' bioaccessibility. Prospecting the antioxidant capacity of soluble digested fraction and the potential prebiotic effect from the undigested material aims to promote a sustainable solution for the agroindustry through the full reuse of broken kernels.

2. Material and methods

2.1. Material

Cashew nut kernels were acquired in the city of Fortaleza (CE/Brazil). Isolated commercial culture of probiotic bacteria was supplied by Sacco (*Lactobacillus plantarum* BG112, recently re-classified as *Lactiplantibacillus plantarum* BG112 according to Zheng et al. (2020), and *Bifidobacterium animalis* BLC1) and Christian-Hansen (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* subspecie *lactis* BB-12). The list of main reagents and chemicals is given in the supplementary data (Table SD1).

2.2. Methods

2.2.1. Sample preparation

Semi defatted cashew nut flour (SCF) was obtained by breaking whole cashew nut kernels in a food processor (Skymsen) and by a cold pressing oil extraction in a manual hydraulic press (model-C, Carver Inc, Indiana, USA) followed by grinding it into a 5 mm mesh. The SCF was submitted into a second oil extraction by n-hexane solvent in a Soxhlet system and then grinding this into a 5 mm mesh in order to produce the defatted cashew nut flour (DCF).

2.2.2. Simulated gastrointestinal digestion (GID)

In vitro gastrointestinal digestion was performed according to Minekus et al. (2014), with slight modifications in the oral phase period as the final ratio sample:digestive fluids and enzymes in each digestion phase. Briefly, DCF (5 g) was added to deionized water (20 mL) prior to the simulated salivary fluid (SSF), aiming to enable α-amylase (75 U/mL) dispersal in the oral phase (pH 7, 10 min). Then, the pH was lowered to 3.0 through the addition of HCl 6 M and a final ratio of DCF to SSF was 17:83 (w/v). The oral bolus was followed to gastric phase, with simulated gastric fluid (SGF) and pepsin (2.000 U/mL) incorporation. This phase was conducted for 120 min and a final ratio of oral bolus to SGF of 75:25 (v/v). The intestinal phase was started by adjustment to pH 7.0 with NaOH 1 M. The simulated intestinal fluid (SIF) was also inserted, as were the enzymes (porcine pancreatin (100 U/mL) and porcine bile extract (10 mM) for 120 min. A final ratio of gastric chyme to SIF of 67:33 (v/v) was obtained. Four trials of the simulated *in vitro* gastrointestinal digestion (GID) were performed in water bath at 37 °C under constant stirring (90 rpm). In order to stop the hydrolysis, the total digested volume was heated to 90 °C for 15 min, cooled down to 4 °C and then centrifuged (3645*g*, 30 min at 4 °C). The defatted cashew nut kernel digested soluble and insoluble fractions (CDs and CDi, respectively) were collected and freeze-dried. The same protocol was conducted without the sample additions (blank), to be discounted from other assay results.

2.2.3. Proximal composition and total phenolic determination

Proximal compositions, such as moisture, lipids, dietary fiber, protein and ashes were performed according to AOAC methods (Latimer & George, 2012), lipids according to AOCS (Collison, 2017), and carbohydrates were determined by difference. Amino acid was separated and quantified in a reversed-phase liquid chromatograph using a Phenomenex LUNA 100 Å (5 μm, 4.6 mm × 250 mm) C18 analytical column and a DAD detector at 324 nm (White, Hart, & Fry, 1986) and LabSolutions series workstation class VP (Kioto, Japan, 2000). The amino acid score was determined according to WHO/FAO/UNU (2007).

Total polyphenols were extracted, as previously described by Kim, Jeong, and Lee (2003), using three solvent extractors (methanol/water

(70:30); ethanol/water (70:30) and distilled water). DCF (100 mg) was added to each type of solvent (4 mL) and one-minute mixed was carried out through Ultra-Turrax T-25, and then centrifuged (1125*g*, 10 min, 4 °C). The supernatant was filtered through Whatman n° 2 paper into a 10 mL volumetric flask and the extraction was repeated once. Polyphenol extracts (300 μL) were added to 60 μL of 2,2,2-trichloroacetic acid (TCA) aqueous solution (10% w/v), incubated 10 min at 4 °C and centrifuged (17,949*g*, 5 min). The supernatants were used for the determination of total phenolic compounds by the Folin-Ciocalteau method, using gallic acid standard solutions for calibration curve (20–120 μg/mL). Results were expressed as mg of gallic acid equivalents (GAE) per g of sample (Al-Duais, Müller, Böhm, & Jetschke, 2009). The polyphenol extracts without TCA addition were also tested.

2.2.4. Molecular weight (Mw) distribution

The DFC and CDs molecular weight profile distribution was performed by Size-Exclusion Fast Protein Liquid Chromatography, SE-FPLC (ÄKTA Pure 25, GE Healthcare, Chicago, Illinois, USA) and columns Superdex 30 Increase 10/300 GL (GE Healthcare, Chicago, Illinois, USA) equipped with a 280 nm UV detector and the Unicorn 6.3 Software. The sample was solubilized (5 mg/mL) in a sodium phosphate buffer (25 mM pH 7,4 with 150 mM NaCl), sonicated for 10 min and filtered through 45 μm PTFE membrane before injection (500 μL). The isocratic 0.5 mL/min flow rate was monitored at 280 nm for both samples and their molecular weight were determined by comparison of their retention times with the retention times of molecular weight standards used, such as α-lactalbumin (14,178 Da), Insulin (5807.6 Da), Vitamin B12 (1355.37 Da), and L-β-4-dihydroxyphenylanine (197.2 Da) (Vander Heyden, Popovici, & Schoenmakers, 2002).

2.2.5. Antioxidant capacity (AC) by in vitro methods: ORAC, ABTS, DPPH and DNA protective 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 described by Chisté et al. (2011). The fluorescence decay was read in a 96-well microplate fluorescence reader (Synergy, BioTek®, Gen5 software), with fluorescence filters for excitation at 485 nm and for emission at 528 nm at 37 °C. A trolox standard curve (12.5–400 μM) was used to express the results as μmol Trolox equivalent/g sample.

2.2.5.2. ABTS. $ABTS^{\bullet+}$ radical (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) scavenging capacity was performed as Al-Duais et al. (2009). A 20 μL aliquot of each extract was put into a microplate well and 220 μL of ABTS working solution (Abs 0.7 ± 0.02) was added to react over 6 min. After, the absorbance was read at 730 nm in UV–Vis microplate Synergy reader (BioTek®, Gen5 software). A trolox standard curve (12.5–200 μM) was built to express the results as μmol Trolox equivalent per g of dry sample. The appropriate solvent blank (240 μL of sodium phosphate buffer) was performed.

2.2.5.3. DPPH. 2,2-Diphenyil-picrylhydrazyl (DPPH) radical scavenging capacity was performed according to Al-Duais et al. (2009). 134 μL of ethanolic DPPH solution 150 μM was added to 66 μL of extract and the reaction mixture was kept in darkness for 45 min before the absorbance was read at 517 nm in UV–Vis microplate Synergy reader (BioTek®, Gen5 software). Appropriate solvent blank (200 μL of ethanol) and solvent control (66 μL of ethanol) were performed. A trolox standard curve in ethanol was made and the antioxidant capacity was calculated as μmol trolox equivalent per g of sample.

2.2.5.4. DNA protective capacity. The ability to avoid DNA strand breakage from ROS action was assessed, as described by Yarnpakdee, Benjakul, Kristinsson, and Bakken (2015). Supercoiled plasmid pcDNA-FLAG (125 ng/mL, 4 μL), dissolved in Tris-HCl-EDTA (TE) buffer (10 mM Tris-HCl and containing 0.1 mM EDTA), was prepared according to Pavan et al. (2016) and mixed to 2 μL of sample aqueous extract (DCF or CDs) and 4 μL of aqueous 2.2′-azobis(2-amidino-propane) dihydrochloride (AAPH) solution 30 mM in a DNAse free microtube at this order. The mixture was incubated in darkness at 37 °C for 1 h. Two controls were performed at the same conditions using ultrapure water instead of AAPH, and sample extract (6 μL, DNA positive control) and sample extract (2 μL, oxidized DNA control). After incubation, the volume (10 μL) was loaded onto 0.8% agarose gel and DNA bands were stained with 1:20.000 SYBR safe (Thermo Scientific) in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate with 1 mM EDTA). Electrophoresis was conducted at 80 mV for 90 min, followed by 120 mV for 60 min, using a horizontal gel electrophoresis system (Bio-Rad, California, USA). A UV light at ChemiDoc Imaging System (Bio-Rad, California, USA) was used to visualize the DNA bands. The quantification was performed in Image J software (NIH, Bethesda, Maryland, USA). The protective effect of extracts was measured by the retention percentage of supercoiled DNA, calculated according to the following Eq. (1).

$$Retention\ \ supercoiled\ DNA\ band\ (\%) = \frac{intensit\ of\ sample\ supercoiled\ band}{intensit\ of\ control\ supercoiled\ band} \times 100 \quad (1)$$

2.3. Potential prebiotic effect

The potential prebiotic effect of insoluble fraction (CDi) after simulated digestion of DCF was determined, as described by Moreno-Vilet et al. (2014) by screening its impact on the growth of four commercial probiotic strains (*Lactiplantibacillus plantarum* BG112, *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subspecie *lactis* BLC1 and *Bifidobacterium animalis* subspecie *lactis* BB-12) in De Man, Rogosa, Sharpe (MRS) medium. All MRS media were prepared by their individual components in order to control the carbohydrate source and content. The standard MRS medium (a positive control, MRS_{pc}) contains sodium acetate (1 g), agar (3 g), dibasic ammonium citrate (0.4 g), peptic digest of animal tissue (peptone A, 2 g), beef extract (2 g), yeast extract (1 g), potassium phosphate (0.4 g), magnesium sulfate (0.02 g), manganese sulfate (0.01 g), 200 μL of polysorbate 80 and dextrose (4 g) diluted in 200 mL deionized water. The dextrose replacement by CDi (3.77 g) in the MRS medium constituted the $MRS_{C\text{-}Di}$. Two more MRS media were performed as controls: adjusted positive control (MRS_{apc}, 1.89 g dextrose) and inulin control (MRC_{ic}, 1.89 g inulin) aiming for the same carbohydrate content as $MRS_{C\text{-}Di}$ (according to its physicochemical composition) in dextrose and in a well-established prebiotic source. All bacteria growth was compared to a MRS medium with no dextrose addition (negative control, MRS_{nc}). Serial dilution of each freeze-dried commercial probiotic bacteria was made in peptone water 0.1% (w/v). The BB-12 and BCL-1 were inoculated in 1×10^9 cfu/g and incubated in anaerobiosis (AnaeroGen®), at 37 °C for 72 h. On the other hand, LA-5 and BG-112 were inoculated in 1×10^{10} cfu/g and incubated in microaerophilia, at 37 °C for 72 h. The bacteria growth was determined through the spread plate technique expressed as log cfu/g.

2.4. Statistical analysis

Data collected from experiments were in triplicate, expressed as means ± standard deviation (SD) and assessed by statistical analysis, using a one-way analysis of variance (ANOVA) followed by Tuckey's post hoc test to analyze if a significant difference ($p < 0.05$) occurred by means of physicochemical composition, total phenolic compounds, ORAC, ABTS and DPPH assay measurements. One-way ANOVA with Dunnet's multiple comparisons test ($p < 0.05$) was performed in DNA protective capacity and prebiotic effect trials. All statistical analyses were carried out using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, CA, USA).

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

Components (%)	SCF	DCF	CDs	CDi
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 SCF: semi defatted cashew nut kernel flour; DCF: defatted cashew nut kernel flour; CDs: soluble digested fraction; CDi: 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 $p < 0.05$.

3. Results and discussion

3.1. Simulated gastrointestinal digestion (GID) and sample characterization

Throughout the gastrointestinal tract, digestive enzymes hydrolyze food into smaller compounds until it is absorbed and reaches its targets (Pimentel et al., 2020). The solubilization of hydrolyzed nutrients makes them more bioaccessible and is an important prerequisite for bioavailability and bioactivity (Cilla et al., 2018). It was therefore important to evaluate the soluble digested fraction for antioxidant properties and the insoluble fraction, for potential prebiotic effect (considered as the one that has a chance of being fermented by the intestinal microbiota). In the DFC sample, the initial content of total solids and protein was of 4.40 g and 1.79 g, respectively. After digestion, 2.69 g of solids (61.19% yield) and 1.25 g of protein (69.71% yield) were solubilized by the action of gastrointestinal enzymes (on a dry basis). The material not solubilized after DCF digestion resulted in a 1.67 g (37.96% yield) of insoluble material (CDi) with the presence of 0.53 g of protein in this fraction.

Nuts usually contain antioxidant phytochemicals, the main ones for cashew nuts being flavonols (catechin and epicatechin and their gallic acid esters), proanthocyanidins, phenolic lignans, and alkylphenols (cardanols, cardes, anacardic acid and their derivatives) (Bodoira & Maestri, 2020). The screening method for total phenolic compounds (Pc) by the Folin Ciocalteau reagent was carried out on the DCF sample with the addition of trichloroacetic acid (TCA) in the extracting solvents that reduce the interference of soluble proteins and large peptides (Greenberg & Shipe, 1979). The Pc content in the aqueous, hydroethanolic (30:70) and hydromethanolic (30:70) extracts were 1.05, 1.44 and 1.77 mg GAE/g DCF, respectively, with no difference between them. Phenolic compounds occur in food in both free and bound form. According to Chandrasekara and Shahidi (2011), the total phenolic compounds in methanolic extract from cashew nuts was 1.14 mg GAE/g and 0.028 mg GAE/g of bound phenolics. Despite the efficiency of methanol, 70% (v/v) ethanol is an interesting choice for extracting bioactive compounds, based on their food quality and safety (Machado et al., 2019). Simple phenolic acids can bind to primary macronutrients, such as proteins and carbohydrates (Alu'datt et al., 2017). Despite the scarce literature, there are reports that dietary fiber reduces phenolic compounds' bioaccessibility in the upper gastrointestinal tract, which leads them to the colon where they might become available through resident microbiota fermentation, releasing metabolites that might be absorbed (Jakobek & Matić, 2019).

Proximal composition (Table 1) showed that DCF has 11.45 g of dietary fiber per 100 g of sample, which has remained in the insoluble fraction (CDi) after digestion (GID). Dietary fibers and other non-digestible carbohydrates may suffer bacterial fermentation when they arrive in the intestine and might modulate gut microbiota (de Andrade et al., 2020).

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

Amino acid (AA)	WHO/FAO/UNU mg/g protein	DCF		CDs	
		mg/g protein	AA score	mg/g protein	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 = glutamic acid, Trp = Tryptophan, Tyr = Tyrosine, Val = Valine. Values are means ± standard deviations of three (3) measurements. np: not applicable.

Therefore, CDi was evaluated as a potential prebiotic substrate. According to Table 1, proteinaceous components are the main SCF and DCF macronutrients and their content was alike those reported in the literature (Liu et al., 2018; Sanchiz et al., 2019). Among the proteins present in cashew nut, previous studies have mainly reported globulin (17.30%) and a similar content of gluten (7.80%) and albumin (7.69%). Moreover, the same studies have concluded that glutelin and albumin are almost fully soluble in neutral pH (Liu et al., 2018) and it probably contributes to the absorption of peptides in the intestine.

3.1.1. Amino acid composition analysis

Cashew nut kernel protein is a high nutritional protein source (Oliveira et al., 2020) that includes all indispensable amino acids (Table 2) despite its low content of lysine, according to the ideal score protein requirement for adult (WHO/FAO/UNU, 2007). Various trials have described different food by-product matrixes as a source of several

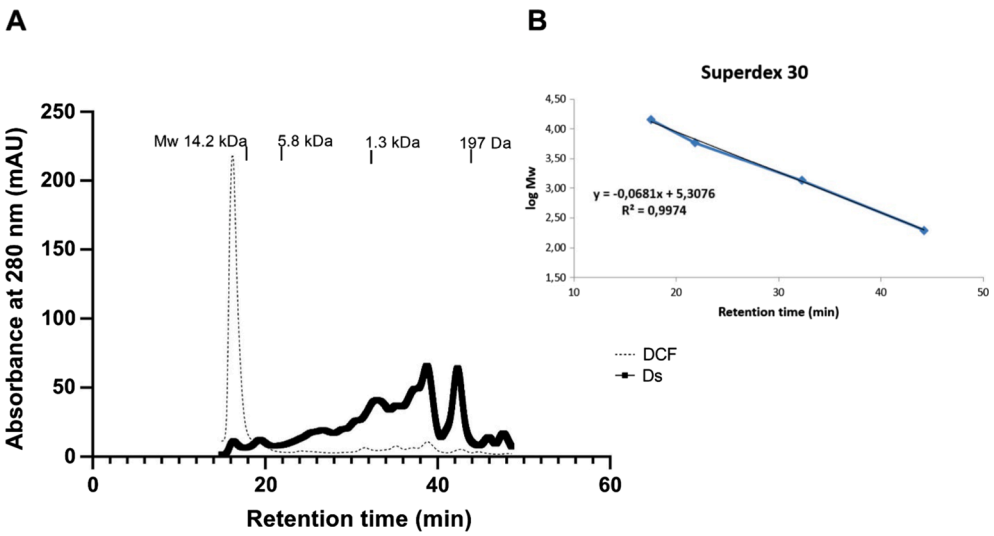

Fig. 1. Molecular weight (Mw) distribution profile by Size-Exclusion Fast Protein Liquid Chromatograph (SE-FPLC). (A) Elution profile of defatted cashew nut flour (DCF; dashed line) and freeze dried digested soluble fraction (Ds; continuous line). (B) The standard linear regression curve for Superdex 30 calibration, generated by plotting the log of the molecular weight (Mw) of standards α-lactalbumin (14,178 Da), insulin (5,807.6 Da), B12 vitamin (1,355.37 Da) and L-β-4-dihydroxyphenylanine (197.2 Da) against their retention time (min).

Table 3
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	CDs
ORAC	Aqueous	$14.8 \pm 0.02^{b, B}$	$526.0 \pm 1.60^{a, A}$
	Ethanolic	$46.3 \pm 1.22^{a, B}$	$101.3 \pm 4.70^{c, A}$
	Methanolic	$39.7 \pm 7.64^{a, B}$	$203.2 \pm 13.58^{b, A}$
ABTS	Aqueous	$5.27 \pm 0.12^{b, B}$	$76.64 \pm 1.58^{d, A}$
	Ethanolic	$10.57 \pm 0.61^{b, B}$	$57.81 \pm 5.87^{de, A}$
	Methanolic	$6.68 \pm 0.78^{b, B}$	$53.32 \pm 1.29^{e, A}$
DPPH	Aqueous	$4.77 \pm 0.60^{b, A}$	$3.61 \pm 2.43^{f, A}$
	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; CDs: defatted cashew nut kernel flour soluble digested 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 ($p \leq 0.05$).

bioactive peptides. They present specific sequences of amino acids that are inactive in the parental protein release after hydrolysis, which can provide health benefits, as antioxidants for instance (Chai et al., 2021; Sánchez & Vázquez, 2017).

Antioxidant peptides usually contain three to sixteen amino acids and a low molecular weight between 0.4 and 2 kDa (Görgüç et al., 2020). Moreover, hydrophobicity and the presence of histidine, glycine, lysine, tyrosine, glutamic acid and sulfur (methionine and cysteine) amino acid residues are important for this bioactivity, as is their sequence (Görgüç et al., 2020; Piovesana et al., 2018). The results showed that hydrophilic amino acids dominate the profile of DCF (50.37%) and CDs (52.87%) while hydrophobic amino acids reach roughly 30% of the DCF and CDs profile. In addition, CDs has a higher concentration of isoleucine, lysine, tryptophan, glycine and glutamic acid after GID, suggesting their presence in soluble peptides. In DCF, proteins are concentrated after oil extraction when compared to raw cashew nut, so the profile presented here is higher than the one recently reported by Liao, Zhao, Xu, Gong, and Jiao (2019).

3.1.2. *Molecular weight (Mw) distribution*

The Mw distribution profile (Fig. 1) shows defatted cashew nut flour (DCF) and defatted cashew nut digested soluble fraction (CDs) compounds that contain aromatic ring (λ 280 nm), such as proteins and phenolic compounds, with no distinction between peptides and phenolic compounds, for example (Albe Slabi et al., 2019). From the DCF curve, 72.43% of soluble components had Mw > 7 kDa, mainly represented by the intense pick below 21.48 min of retention time. This indicates the probability of unhydrolyzed proteins, glutelin, albumin and globulin, which have high Mw, as described in the literature (Liu et al., 2018; Sathe et al., 2009). Gastrointestinal digestion releases peptides from proteins and it is well established that the Mw of hydrolysate is crucial for their biological activity. Since soluble low Mw peptides are easily absorbed in the gastrointestinal tract and promptly reach the cardiovascular system to exhibit physiological-regulation properties (Rani, Pooja, & Pal, 2018). Hence, CDs show a large area in latter running time since 76.90% of soluble components had 0.1 kDa < Mw < 2 kDa.

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

Antioxidant measurements (Table 3) for DCF show a better effectiveness of the hydroalcoholic extracts in ORAC assay, resulting in higher antioxidant capacity (Ac), without difference between solvents used ($p > 0.05$). These results are lower than the respective ones quantified by Chandrasekara and Shahidi (2011) in methanolic 80% (v/v) extract for ORAC, ABTS and DPPH per g of defatted meal (3207 TE μmol/g; 3.17 mg of GAE/g and 38.9 μmol of TE/g, respectively). Although, they were similar to what was recently reported for raw cashew nut by Sanchiz et al. (2019). Surprisingly, after the simulated digestion, the antioxidant capacity of the soluble fraction was higher than the flour in the ORAC analysis, and more intense in the aqueous extract, followed by methanolic and ethanolic extracts.

The present results reveal higher ($p \leq 0.05$) ORAC and ABTS values for CDs, compared to DCF. The GID probably releases phenolic compounds which are bound to protein fractions and antioxidant peptides, which are more available to interact to reactive oxygen species (ROS) and develop a higher antioxidant capacity. It is important to evaluate the structure of the peptides to ensure their antioxidant bioactivity, since amino acid sequence and composition is of utmost relevant to develop bioactivity (Pimentel et al., 2020; Rodríguez & Tironi, 2020). However, an opposite behavior was observed for the DPPH assays, where the sample CDs resulted in smaller antioxidant capacity when compared to DCF. This probably occurred because of the difference between the characteristics of the radicals DPPH and ABTS, since the last test promoted better results for hydrophilic compounds (Menghini et al., 2018).

Among the antioxidant defense pathways, the most accepted mechanism is the elimination of ROS, which can be carried out both by

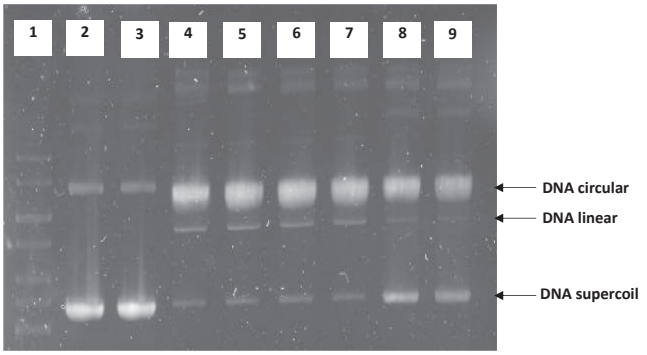

Fig. 2. Agarose gel electrophoresis of DNA supercoil band protective capacity assay. The numbers refer to 1: Molecular weight standard; 2 and 3: DNA positive control; 4 and 5: DNA negative control; 6 and 7: DNA + AAPH + DCF; 8 and 9: DNA + AAPH + CDs.

transferring the hydrogen atom (e.g., ORAC and inhibiting lipoperoxidation) and by transferring electrons (e.g., plasma iron reduction capacity) (FRAP), ABTS and DPPH) (Granato et al., 2018). Therefore, the compounds of the cashew nut may have the ability to act by both antioxidant mechanisms. Further studies are needed to identify responsible bioactive antioxidant compounds (phenolics and peptides). The major contribution was the finding that the set of compounds released after DCF gastrointestinal digestion are likely to develop synergistic activity in antioxidant defenses.

In a biological context, as expected from the DNA's protective capacity assay, Fig. 2 reinforces the increase in antioxidant activity after digestion, shown by the sample CDs (lines 8 and 9) compared to the sample CDF (lines 6 and 7) before GID. The breakdown of the original DNA by the activity of oxidizing agents releases two fragments: one circular and the other linear (Yarnpakdee et al., 2015). The degradation of the supercoiled plasmid pcDNA-FLAG is confirmed by the reduced intensity of the supercoil band in agarose gel electrophoresis (Salar, Purewal, & Sandhu, 2017). Therefore, the acute supercoiled band indicates the greater antioxidant activity of the compounds that protect the DNA molecule from oxidation of the peroxyl radical induced by the thermo-degradation of AAPH. Fig. 2 shows that the retention of the supercoil band in the DNA negative control samples (lines 4 and 5) DCF and CDs was of 5.94%, 5.50% and 29.02%, respectively. Although the digested sample (CDs) show a difference ($p < 0.05$) from the DNA control sample (lines 2 and 3), the GID partially promoted the antioxidant capacity of DCF. Studies have pointed out that the consumption of nuts, such as almonds, hazelnuts, pistachios, walnuts, and cashew nuts is positively correlated with the reduction of many chronic diseases, possibly because they help reduce oxidative damage (Rocchetti et al., 2019; Silva et al., 2017).

3.3. Potential prebiotic effect

The potential prebiotic effect of cashew nut kernel flour was investigated (Fig. 3) by replacing the carbon source in the culture medium by an ingredient of interest, similar to other published papers (de Andrade et al., 2020; Moreno-Vilet et al., 2014).

Contrary to expectations, all probiotic strains evaluated showed high growth in the culture medium formulated without the carbon source (negative control, MRSnc), possibly due to the adaptation to use other nutrients contained in the culture medium as a source of energy. Even so, a higher growth ($p < 0.05$) was observed for *B. animalis* BB-12 in the MRS medium added to CDi as an energy source, when compared to the negative control, pointing to a potential prebiotic effect of the cashew nut kernel flour. However, an equivalent growth promotion was not observed in the other probiotic cultures evaluated, nor was it in a second strain of *B. animalis* (BLC-1) evaluated.

Phylogenetic differences between bacteria may reflect their ability to use a carbohydrate source as a growth factor (Moreno-Vilet et al., 2014), therefore other bacterial species and genus should also be tested. Despite promoting bacterial proliferation, proteinaceous substract are not categorized as prebiotic substracts (Gibson et al., 2017) so protein content has no prebiotic effect and it may have contributed to a high bacteria count plate in the negative control. Dietary fibers' CDi content may be the most relevant compounds for the potential prebiotic effect observed, although phenolic compounds should also be investigated in future studies since they were recently included as prebiotic substrates (Gibson et al., 2017). Proanthocyanidins (polymerized polyphenol) are able to be fermented by colonic microbiota, releasing several low molecular weight phenolic metabolites (Rocchetti et al., 2019), which in turn can develop a prebiotic effect (Sugizaki & Naves, 2018). Other *in vitro* or *in vivo* analyses could be used to investigate the prebiotic effect, such as optical density, culture medium pH mesurements, fecal sample analysis, *in vitro* fermentation models to mimic the large intestine's physiological environment, apart from antipathogenic effects before clinical trials (Narendra Babu et al., 2018; Rocchetti et al., 2019).

4. Conclusion

The cashew nut flour digested by a procedure similar to human digestion released a pool of compounds, peptides, phenolic compounds and their derivatives, which are likely to act in synergy, resulting in high antioxidant and DNA protection action. The action of digestive enzymes promoted the hydrolysis and release of many soluble compounds of low molecular weight that are more bioaccessible to interact with reactive species.

In addition, the insoluble fraction (CDi) applied in the MRS culture

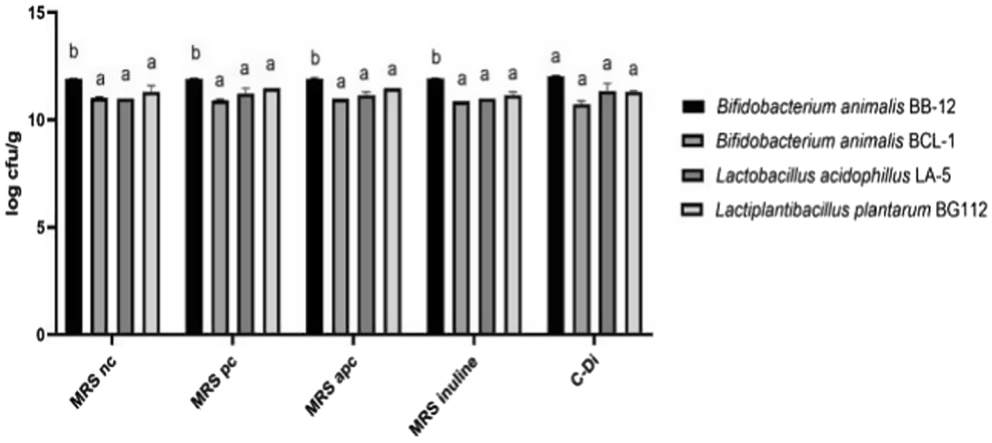

Fig. 3. Potential prebiotic effect. Bacteria growth in each MRS medium tested were performed in duplicate. The values are the means ± standard deviation and expressed as log cfu/g. Data with different superscript letters are different to MRSnc at $p < 0.05$ according to one-way ANOVA and Dunnett pos hoc test. Abbreviations include: MRSnc (negative control, without carbohydrate source), MRSpc (standard positive control, 4 g dextrose) MRSapc (adjusted positive control, 1.89 g dextrose), MRSinulin (prebiotic control, 1.89 g inulin) and C-Di (MRS medium with CDi as carbohydrate source).

medium as a carbohydrate substitute, had a bifidogenic effect for *Bifidobacterium animalis* subs. *lactis* BB-12. Therefore, considering the matrix digestion process and the set of released compounds, they suggest the potential of this co-product for human health. Its potential for application by the cashew nut industry for the development of functional foods was evidenced, enabling the total reuse of the by-product. However, future research must be done to identify the bioactive compounds and their bioaccessibility in the application of specific products, even by *in vitro* or *in vivo* methods.

CRediT authorship contribution statement

Mariana Sisconeto Bisinotto: Investigation, Methodology, Formal analysis, Investigation, Writing - original draft. **Daniele Cristina da Silva:** Investigation. **Luciana de Carvalho Fino:** Investigation. **Fernando Moreira Simabuco:** Investigation, Formal analysis, Writing - review & editing. **Rosângela Maria Neves Bezerra:** Investigation, Funding acquisition, Writing - review & editing, Supervision, Resources. **Adriane Elisabete Costa Antunes:** Conceptualization, Writing - review & editing, Supervision, Resources. **Maria Teresa Bertoldo Pacheco:** Conceptualization, Project administration, Funding acquisition, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109906.

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