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Organic arabic coffee husk: Antioxidant and cytoprotective properties and potential impacts on selected human intestinal bacterial populations of individuals with diabetes

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ABSTRACT

Coffee husks are the main by-product of the coffee industry and have been traditionally discarded in the environment or used as fertilizers. However, recent studies have shown that coffee husks have bioactive compounds, such as phenolics and fiber-bound macro antioxidants, offering a range of potential health benefits. This study evaluated the antioxidant capacity, cytoprotective/cytotoxic properties, and stimulatory effects on the relative abundance of selected intestinal bacterial populations of individuals with diabetes of organic coffee husks. Organic coffee husk had good antioxidant capacity, maintained under simulated gastric conditions, with more than 50% of antioxidant capacity remaining. Organic coffee husk exerted cytoprotective properties in Caco-2 cells, indicating that cellular functions were not disturbed, besides not inducing oxidation. Overall, organic coffee husk promoted positive effects on the abundance of distinct intestinal bacterial groups of individuals with diabetes during *in vitro* colonic fermentation, with a higher relative abundance of *Bifidobacterium* spp., indicating the availability of components able to reach the colon to be fermented by intestinal microbiota. Organic coffee husk could be a circular material to develop new safe and pesticide-free functional ingredients with antioxidant and potential beneficial effects on human intestinal microbiota.

1. Introduction

Coffee is a foodstuff that is traded and consumed all over the world. The world's largest producer is Brazil, where the Arabica variety is grown on a large scale, either conventionally or organically, which provides safer fruit with a different chemical composition for consumption. However, the processing of coffee produces an excessive amount of by-products that can have a negative impact on the environment. In this way, sustainability throughout the coffee processing process becomes a fundamental issue that aims to minimize these

Abbreviations: RAC, Remaining Antioxidant Capacity; AnaeroGen, Anaerobic Generator system; APPH, 2,2′-azobis(2-amidinopropano) dicloridrato; AUC, Area Under Curve; CH, Coffee Husk; CHE, Coffee Husk Extract; DCF, 2′,7′–dichlorofluorescein; DCF-DA, 2′,7′-Dichlorodihydrofluorescein Diacetate; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethylsulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, Ethylenediaminetetraacetic Acid; FBS, Fetal Bovine Serum; FRAP, Ferric Reducing Antioxidant Power; HPLC, High Performance Liquid Chromatography; H_2O_2, Hydrogen Peroxide; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide; NC, Negative Control; ORAC, Oxygen Radical Absorbance Capacity; PBS, Phosphate Buffered Saline; ROO*, Peroxyl Radical; ROS, Reactive Oxygen Species; TEAC, *Trolox* Equivalent Antioxidant Capacity; UV–VIS, Ultraviolet–Visible Spectroscopy.

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environmental impacts, which are mainly generated by the coffee husks, which are the most common by-product of this process (Cordoba et al., 2020; Silva et al., 2024).

Organic coffee husks are rich in polyphenols, compounds that have a wide range of biological effects beneficial to human health, making these husks a viable source for reuse as they contain bioactive components (Marques et al., 2022). Among the various effects of phenolic compounds, the most reported is their antioxidant capacity, but also their association with beneficial metabolic effects such as the regulation of blood glucose in people with type 2 diabetes (Abreu et al., 2024; Mithul Aravind et al., 2021; Das & Gnanasambandan, 2023).

However, it should be noted that to validate the physiological benefits of phenolic compounds from any food matrix, their transformations and behavior in the human gastrointestinal tract must be specifically studied, as they are unstable to the digestive process, which is a factor that can reduce their bioavailable concentrations in the intestine and their bioavailability and consequently their effect in the body (Silva et al., 2024). Phenolic compounds ingested with food have the ability to exert antioxidant effects both in the gastrointestinal tract and at the systemic level after being absorbed by intestinal cells (Grzelczyk et al., 2022). The antioxidant capacity of a compound is considered extremely important for human health. To evaluate this effect on compounds released from matrices such as organic coffee husks, several *in vitro* methods with different specificities are used, such as Reducing Antioxidant Power Ferric Acid (FRAP), Oxygen Radical Absorbance Capacity (ORAC), and Free Radical Inhibition 2,2-diphenyl-1-picrylhydrazyl (DPPH), which must be used together given the complex nature of the compounds of each matrix (Finotti et al., 2024). Other possible biological effects of phenolic compounds can also be evaluated through the *in vitro* use of Caco-2 cells, a cell line used as a cell model for the intestinal barrier that allows the study of bioavailability by evaluating the absorption, transport, and metabolism of the compounds, as well as the cytotoxicity and cytoprotective effects of these components (Grzelczyk et al., 2022).

In addition to the above effects, phenolic compounds also have the ability to promote health as they are potential substrates for microorganisms in the human gut microbiota. It is reported that a significant proportion of digested phenolic compounds are not absorbed in the small intestine. In this way, these compounds reach the large intestine, where they are metabolized by the microorganisms of the large intestine, leading to a beneficial modulation of the gut microbiota population and generating more active secondary metabolites that can be of great benefit to the host (Gibson et al., 2017; Massa et al., 2022). These effects can be evaluated in phenolic compounds released from industrial food processing by-products, such as coffee fruit peels, through experimental *in vitro* models that simulate fermentation by the gut microbiota by using a fermentation with human feces (Menezes et al., 2021).

Considering the above context, it is suggested that organic coffee husks are promising as a food matrix that can benefit human health and therefore should be better evaluated for objective and effective reuse. However, it is clear that for the consolidation of this matrix as a health-promoting food, a more in-depth study of the bioactive effects of this matrix and its phenolic compounds is required.

Therefore, the aim of this study was to investigate the antioxidant capacity of organic coffee husks before and after gastrointestinal digestion *in vitro*, the effects on the viability and cell protection of Caco-2 cells, and the effects on the fecal microbiota of type 2 diabetic patients in an *in vitro* fermentation system.

2. Material and methods

2.1. Coffee husk

Organic coffee husk (100 % Arabica and typical) was collected from a private farm (Taquaritinga do Norte, PE, Brazil; −7.88809 S, 36° 5′ 33″ W), during the 2022 harvest. The organic coffee was produced with shading technique and harvested by hand. After coffee wet processing, the husk was separated from the grain and frozen at −18 °C.

2.2. Sample preparation: Coffee husk flour

The coffee husk was dried in a forced-circulation oven at 40 °C for 48 h using previously described conditions (Silva et al., 2020). Dried coffee husks were ground to 2 mm with a Wiley knife to form a fine bran-like mass and stored in a hermetically sealed package protected from light under refrigeration (5 °C).

2.2.1. Preparation of the organic coffee husk extract (CHE)

The coffee husk flour (CH) was weighed directly into a Falcon tube and homogenized manually with an extraction solution (water + ethanol, 1:1) for 5 min. The ratio of flour to extraction solution was 1:10 (mass:volum). The resulting mixture was incubated at 60 °C in a water bath for 60 min, centrifuged (3500*g*, 20 min, 10 °C), and the supernatant was collected and filtered with a filter paper. The resulting extract was rotary evaporated (Fisatom 802, São Paulo, SP, Brazil) at 60 °C for 5 h and reconstituted with ultrapure water to obtain the original extract volume (Silva et al., 2020).

2.3. Cytotoxic/cytoprotective assays

2.3.1. CACO-2 cell culture

Cells were seeded in 75 cm^2 flasks and grown in DMEM supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin, 8.4 mM Hepes, 1 % sodium pyruvate, 1 % non-essential amino acids, and 1 % L-glutamine. The medium was replaced every 2–3 days. Exponentially growing cells were detached from the culture flasks using 0.05 % trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded on 98-well plates with $2x10^4$ cells per well. After reaching confluence, the growing medium was removed, and cells were pre-treated with control or different concentrations of coffee husk extract (CHE) (1, 10, 100, 500, and 1000 mg/ml) for 3 h, and the cells were stimulated with control or hydrogen peroxide (H_2O_2) at a concentration of 1 mM for an additional 2 h. The cells in the control group were treated with a medium free of FBS. After these procedures, the cell viability assay was performed, and ROS production was measured.

2.3.2. Determination of the effects of CHE on the viability of Caco-2 cells

The evaluation of the viability of Caco-2 cells was based on the cellular uptake of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide (MTT, M5655, Sigma-Aldrich St. Louis, MO, USA), which is endocytosed, and mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals insoluble in aqueous solutions. Cells were incubated with 50 μg/ MTT during the last 30 min of treatment at 37 °C, the medium was removed, MTT crystals were dissolved in DMSO, and absorbance values were measured at 560 and 650 nm. The reduction of MTT was calculated as [(abs at 560 nm) − (abs at 650 nm)] and expressed as a percentage of baseline measurements.

2.3.3. Evaluation of the effects of CHE on the production of reactive oxygen species in Caco-2 cells

The ROS production in Caco-2 cells was evaluated using DCF-DA assay, which is based on the diffusion of DCF-DA into the cells, deacetylation by cellular esterases, and oxidation by ROS into 2′,7′–dichlorofluorescein (DCF). DCF-DA is a fluorogenic dye that measures the activity of reactive oxygen species (ROS) within the cell. In the last 30 min of pre-treatment, cells were incubated with 20 μM of DCF-DA. After the pre-treatment medium was removed, the cells were washed with PBS to remove DCF-DA not internalized and treated with medium (control group), examined CHE concentration, or stimulus plus CHE concentration. The fluorescence was measured immediately during 120 min. The AUC (area under curve) was calculated, and the difference

between sample AUC and control AUC was used as the selective basis of the co-incubation time of DCF-DA and pure treatment with Caco-2 cells.

2.4. Evaluation of the antioxidant activity of CHE in vitro and cell model

2.4.1. DPPH and FRAP assays

The ability of CHE to scavenge the DPPH radical was determined using a previously described method (Brand-Williams et al., 1995). The radical scavenging activity and antioxidant capacity of CHE were measured using a UV–VIS spectrophotometer (Quimis, São Paulo, SP, Brazil) at 515 nm.

The antioxidant ability of CHE to reduce iron (Fe^{3+}) to the ferrous form (Fe^{2+}) – FRAP, was verified at 593 nm in a UV–VIS spectrophotometer (Quimis, São Paulo, SP, Brazil) (Benzie & Strain, 1999). Calibration curves were prepared with different concentrations of Trolox (50–1000 μM), and the results were expressed as equivalent Trolox antioxidant activity per gram of sample.

2.4.2. ORAC assay

The ORAC assay is based on the ability to reduce the peroxyl radical (ROO*) generated by the thermal degradation of 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and protect the fluorescein molecule from the action of the peroxyl radical by the antioxidant fluorescein. The fluorescence decay is monitored in kinetic mode with readings per min for 2 h (pH 7.4) (Dávalos et al., 2004). Analysis was performed in a 96-well microplate fluorescence reader (Synergy, BioTek®, Gen5 software) with fluorescence filters for excitation at 485 nm and emission at 528 nm at 37 °C. A standard Trolox curve was used to express the ORAC values of the samples at concentrations of 250, 500, and 1000 mg/L. A 75 mM potassium phosphate buffer (pH 7.4) was used for curve dilution and sample substrate. In each well of the microtiter plate, 30 μl of sample or standard, 60 μl of 508.25 nM fluorescein solution, and 110 μl of 76 mM AAPH solution were added. Sample and protective standard (AUCnet) were calculated by the difference between the area under the fluorescence decay curve of sample/standard (AUC sample/standard) and the area under the fluorescence decay curve without sample or addition of Trolox (white AUC). Results were expressed as μmol Trolox equivalent/g sample (Dávalos et al., 2004).

2.5. In vitro gastrointestinal digestion of CH and evaluation of the remaining antioxidant capacity

The *in vitro* gastrointestinal digestion of CH was performed as previously described (Brodkorb et al., 2019). Aliquots of CH were prepared to determine the effects of the *in vitro* gastrointestinal conditions on its antioxidant capacity. Stock solutions of electrolytes and enzymes comprising the oral, gastric, and intestinal phases were prepared in accordance with an international consensus (INFOGEST). Antioxidant capacity remaining (RAC) after *in vitro* digestion was expressed as a percentage and determined according to Equation 1(de Oliveira Ribeiro et al., 2020).

$$\text{RAC (\%)} = (\text{CH digested/CH undigested}) \times 100 \quad (1)$$

Where: *CH digested* is the concentration of the analyzed compound from digested CH (intestinal phase), and *CH undigested* is the concentration of the analyzed compound from undigested CH (initial).

2.6. Human fecal inoculum and in vitro colonic fermentation of CH

Fecal samples from four adult volunteers with type 2 diabetes (two men and two women, aged between 25 and 55 years) were used after approval by a Human Research Ethics Committee (Federal University of Paraíba, João Pessoa, PB, Brazil, CAE number: (65756222.4.0000.5188) approved on February 23, 2023. Inclusion criteria included diagnosed type 2 diabetics taking medications to control blood glucose levels, who did not suffer from any gastrointestinal or colonic disease, following a regular omnivorous diet without using probiotic foods, concentrated probiotics or prebiotics in the last 7 days and antibiotics during the six months prior to the study. Donors received specific instructions as well as an appropriate collection kit. The stool collection kit included sterilized collection tubes, disposable spatulas, instructions for sample collection/sending, disposable gloves, and 70 % alcohol. The instructions contained in the kit described the steps for hand hygiene and aseptic handling of the samples. The samples were placed in a jar with an anaerobic generator system (AnaeroGen, Oxoid, Basingstoke, England) and transported to the laboratory. The experiments started immediately after the arrival of the feces to guarantee a fresh fecal microbiota, and the feces were manipulated in an anaerobic environment. The same amount of feces from each donor was pooled (1:1:1:1 w/w) and mixed with sterile phosphate-buffered saline (PBS; 0.1 M; pH 7.4; 1:10, w/v) under agitation (200 rpm) for 2 min for use in the experiments. As the focus of the study was on individuals with type 2 diabetes, all procedures considering the aspects related to the intestinal microbiota were conducted exclusively within this group.

The colonic fermentation was performed as previously described (de Albuquerque et al., 2021)using 40 % (v/v) of autoclaved (121 °C, 1 atm, 15 min) fermentation medium [4.5 g NaCl, 4.5 g KCl, 1.5 g $NaHCO_3$, 0.69 g $MgSO_4$, 0.8 g L-cysteine, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salt, 0.08 g $CaCl_2$, 0.005 g $FeSO_4$, 1 mL Tween 80, 4 mL resazurin solution (0.25 g/L, as an anaerobic indicator) and 1L of distilled water], 40 % (v/v) of the pooled fecal inoculum, and 20 % (w/v) of the digested CH (Brodkorb et al., 2019). Fermentation was carried out at 37 °C for 48 h under anaerobic conditions (AnaeroGen). The CH was previously submitted to an *in vitro* gastrointestinal digestion using the INFOGEST protocol. The fermentation medium without adding CH corresponded to a negative control.

2.7. Evaluation of the relative abundance of intestinal bacterial groups during CH in vitro colonic fermentation

The relative abundance of selected intestinal bacterial groups was measured using fluorescent *in situ* hybridization coupled with flow cytometry using four distinct probes (oligonucleotides) labeled with fluorescent Cy3 and capable of hybridizing the specific region of the 16S rRNA gene of *Lactobacillus* spp./*Enterococcus* spp. (probe Lab 158, facultative anaerobes), *Bifidobacterium* spp. (probe Bif 164, strictly anaerobic), *Bacteroides* spp./*Prevotella* spp. (probe Bac 303, strictly anaerobic), and *Clostridium histolyticum* (probe Chis 150, strictly anaerobic) (Sigma-Aldrich). The total bacterial population was quantified using SYBR Green staining (Invitrogen, Carlsbad, CA, USA) (Conterno et al., 2019). These target human intestinal bacterial groups were selected because alterations in their relative abundances are typically linked to positive (*Lactobacillus* spp./*Enterococcus* spp. and *Bifidobacterium* spp.) and negative (*Bacteroides* spp./*Prevotella* spp. and *C. histolyticum*) metabolic responses and health outcomes in the host, besides being among the most important fermenting bacterial populations found in the human intestine (de Albuquerque et al., 2020; Massa et al., 2022). Measurements were performed using a BD Accuri C6 flow cytometer (New Jersey, USA) with 488 nm excitation of a solid-state blue laser. Signals from individual cells through the laser zone were recorded as logarithmic signals. Fluorescence signals were recorded in FL1 (SYBR Green) and FL2 (Lab 158, Bif 164, Bac 303, and Chis 150) channels and plotted as cytograms using BD Accuri C6 software. The results were expressed as the relative abundance (%) of each measured bacterial group in relation to the total bacterial population.

2.8. Measurements of phenolic compounds during CH in vitro colonic fermentation

2.8.1. Determination of phenolic compounds during CH in vitro colonic fermentation

The contents of phenolic compounds were determined in freeze-dried CH before and after the *in vitro* gastrointestinal digestion (non-dialyzed fraction). Initially, methanol extracts of CF exposed and non-exposed to the *in vitro* gastrointestinal digestion were prepared using previously described procedures (Massa et al., 2022; Padilha et al., 2017). The separation and quantification of the phenolic compounds were performed with HPLC using an Agilent chromatograph (model 1260 Infinity LC, Agilent Technologies, St. Clara, CA, USA) and analytical conditions previously described (Massa et al., 2022; Padilha et al., 2017). A Zorbax C18 (12.6 mm × 4.6 mm, 5 μm; Agilent Technologies) pre-column and a Zorbax Eclipse Plus RP-C18 (100 mm × 4.6 mm, 5 μm; Agilent Technologies) column were used for the determination of phenolic compounds. The gradient used in the separation was 0–5 min: 5 % B; 5–14 min: 23 % B; 14–22 min: 26 % B; 22–25 min: 80 % B, where solvent A was 0.1 M phosphoric acid solution (pH 2.0) and solvent B was methanol acidified whit H_3PO_4 0.5 %. The detection of compounds was performed at 220 nm for (+)-catechin, (−)-epigallocatechin, (−)-epicatechin gallate, procyanidin B1 and procyanidin B2; 280 nm for gallic acid and syringic acid; 320 nm for caftaric acid, caffeic acid and ρ-coumaric acid; and 520 nm for malvidin 3,5-diglucoside, cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, peonidin 3-O-glucoside and malvidin 3-O-glucoside. The data were processed with OpenLAB CDS ChemStation EditionTM software (Agilent

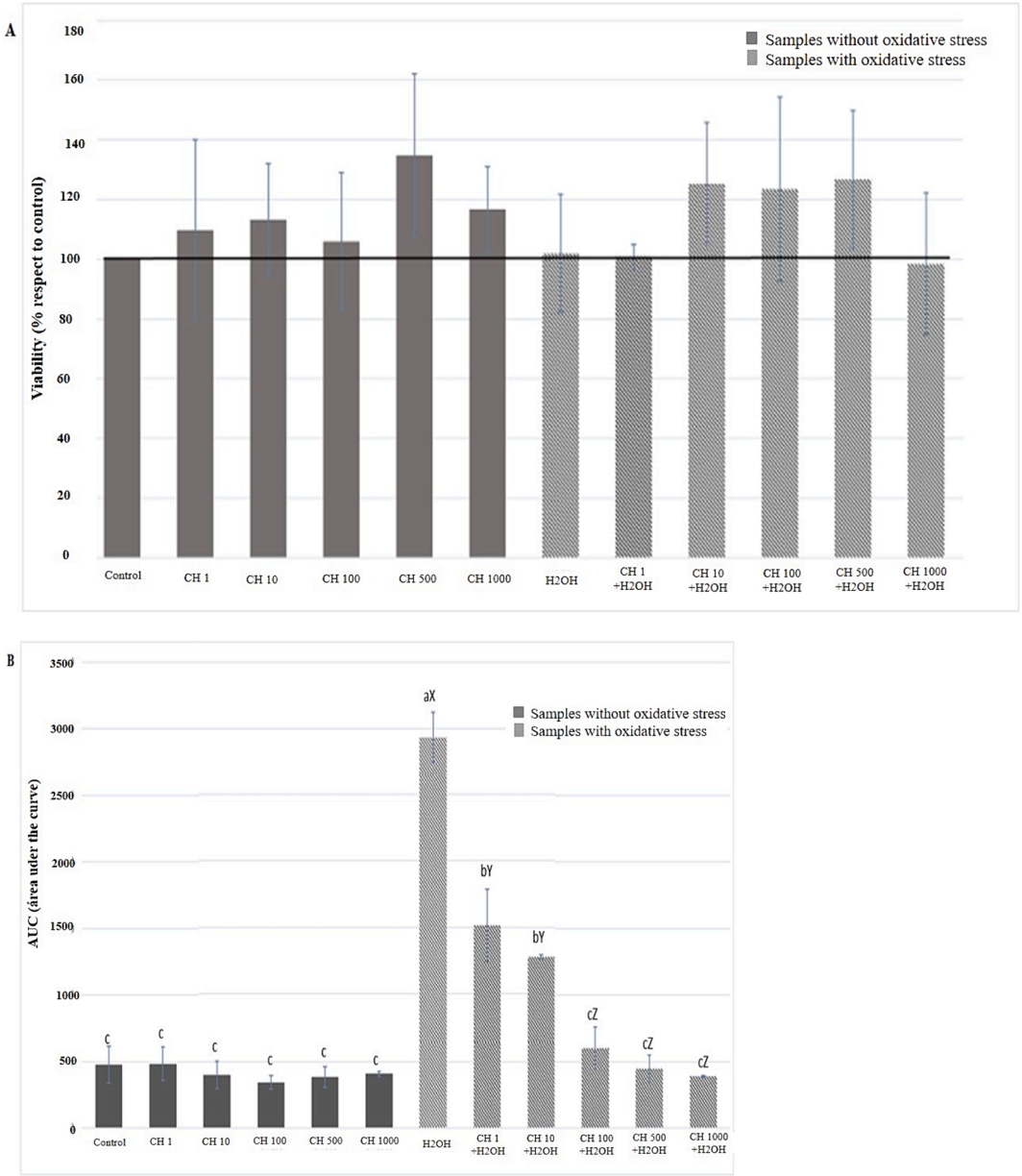

Fig. 1. Cytoprotection and ROS accumulation assay using organic coffee husk extract as a stimulus in Caco-2 cells. **A** – Cell viability test Coffee husk extracts. **B** – Evaluation of (ROS) production in Caco-2 cells. (CH) and H_2O_2 did not affect cell viability. Caco-2 cells were treated at different concentrations of coffee extracts (1, 10, 100, 500, and 1000 μg/ml) with or without oxidative stimulus (H_2O_2 1 mM) for 3 h. Values are expressed as mean ± standard deviation (n = 3). The values did not indicate statistically significant differences ($p < 0.05$). Different letters indicate statistically significant differences ($p < 0.05$). (a, b, c) indicate statistical differences between all samples. The averages referring to the samples without the oxidative stimulus did not differ statistically ($p < 0.05$). (x, y, z) correspond to samples exposed to H_2O_2.

Technologies). The peaks of phenolic compounds were identified by comparison of their retention times with those of external standards (Sigma-Aldrich). The quantification of phenolic compounds was performed with calibration curves of the external standards (R2 of ≥ 0.998) (Menezes et al., 2021). The results were expressed as g/100 g.

2.9. Statistical analysis

The experiments were performed in triplicate on three independent occasions, and the results were expressed as average (*Tukey*) ± standard deviation. The data normal distribution was checked using the Kolmogorov-Smirnov normality test. The results were analyzed using analysis of variance (ANOVA) followed by factor analysis using Minitab® 19 statistical software based on a 5 % significance level. Pearson's correlation test evaluated the relationship between the values of phenolic compounds. GraphPad Prism 6.0 calculation software (GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis.

3. Results and discussion

3.1. Effects of organic coffee husk extract (CHE) on the viability and cytoprotection of Caco-2 cells

The potential toxicity of organic coffee husk extract (CHE) was evaluated by examining the effects of coffee husk extract on the viability of Caco-2 cells using the MTT assay. The results (Fig. 1A) showed that CHE was not toxic to Caco-2 cells, regardless of the concentrations used or incubation with H_2O_2, and did not affect their viability, indicating that the compounds present in CHE do not affect the functions of Caco-2 cells.

Bedoya-Ramírez et al. (2017) showed that treatment of Caco-2 cells with Colombian coffee samples did not promote cell cytotoxicity and, moreover, prevented cell viability from decreasing, confirming our results. Regarding the cytoprotective effect of the DCF-DA assay, treatment of Caco-2 cells with CHE without oxidative stimulation with hydrogen peroxide (H_2O_2) did not significantly alter the level of intracellular reactive oxygen species (ROS) ($p > 0.05$) (Fig. 1B), indicating that the phenolic compounds extracted from CH do not act as oxidizing agents. On the other hand, stimulation of Caco-2 cells with H_2O_2 significantly ($p \leq 0.05$) increased intracellular ROS levels, but these levels were reduced after application of CHE at concentrations of 100, 500, and 1000 mg/mL. Oxidative stress causes the mitochondria present in cells to increase the production of ROS such as H_2O_2, superoxide, superoxide, and hydroxyl free radicals, which can lead to various cellular disorders such as DNA damage, irregular signaling transduction, transduction, and changes in membrane permeability due to lipid oxidation (Liu et al., 2021).

According to Mcdade et al. (2020), the phenolic compounds present in CH are associated with important ROS inactivation actions. This potential may explain the behavior observed in Caco-2 cells treated with CHE, since CHE provides cellular protection against the oxidative action of H_2O_2. This effect isis probably related to its content of phenolic compounds consisting of chlorogenic acids and their derivatives (caffeic and cinnamic acids) and their antioxidant capacity. This effect is of great importance for human health, as an increase in ROS levels in the cells of the intestinal barrier favors the onset of various intestinal pathologies such as Crohn's disease, as well as non-communicable diseases such as obesity and type 2 diabetes (Chen et al., 2020).

In a study by Bedoya-Ramírez et al. (2017), it was shown that treatment of Caco-2 cells with extracts from Colombian coffee samples reduced intracellular ROS levels after the cells had been exposed to oxidative stress with H_2O_2, indicating a cytoprotective effect of coffee components, as in this study. Iriondo-DeHond et al. (2016) reported that free chlorogenic acids may be responsible for the antioxidant capacity of the aqueous extract of coffee silver skin, which, similar to the CHE results of this research, does not promote cytotoxicity to HaCaT cells and also exerts anti-aging properties.

3.2. Antioxidant capacity of organic coffee husks (CH) before and after digestion in the gastrointestinal tract in vitro

The results regarding the antioxidant capacity of CH are presented in Table 1 and show that the extract obtained from CH before *in vitro* digestion had a significant antioxidant capacity against DPPH radicals, in the reduction of ferric ions (FRAP) and in the absorption of oxygen radicals (ORAC), which were 1995.5 μmol TEAC/g, 2043.1 μmol TEAC/g and 65.44 μmol TEAC/g, respectively. In addition, as in our study, Marques et al. (2022) showed that CHE has the potential to delay oxidative effects through different mechanisms of action, the effectiveness being related to the content of phenolic compounds in the CHE observed in the study, which was 24.1 mg CAE/g and consists of compounds such as chlorogenic, gallic and caffeic acids, which are considered powerful antioxidants that help protect the coffee fruit from the deterioration of lipids and proteins due to oxidation processes (Silva et al., 2020).

After the *in vitro* digestion process, the antioxidant potential of CHE changed significantly (Table 1). In the intestinal phase of the simulated gastrointestinal tract, final results of 1139.0 μmol TEAC/g for DPPH, 1883.98 μmol TEAC/g for FRAP and 33.48 μM TEAC/g for ORAC were observed, which were significantly lower ($p < 0.05$) than the values found in the CHE tests before digestion. The decrease in the antioxidant potential of CHE indicates that exposure to gastrointestinal conditions impairs its antioxidant capacity.

According to Mcdade et al. (2020), this reduction in potential is likely due to the oxidation of phenolic compounds under simulated gastrointestinal conditions. In a previous study, Abreu et al. (2024) demonstrated that the bioavailable phenolic compounds after gastrointestinal digestion of CHE *in vitro* had significantly lower concentrations than the concentrations of the compounds before digestion. This result could be related to the decrease in antioxidant capacity observed for CHE in this study, as the concentration of phenolic compounds is directly related to the antioxidant potential present.

Silva et al. (2024) subjected free organic coffee husk extract to *in vitro* digestion in the gastrointestinal tract and observed a decrease in the antioxidant capacity of the extract compared to its potential before digestion, similar to this study.

According to Castaldo et al. (2020), the degradation of compounds during digestion can be caused by changes in pH in the gastrointestinal tract and the action of digestive enzymes in the stomach and intestines. Despite the observed decrease, it is important to emphasize that the remaining antioxidant capacity (RAC) for CHE was more than 50 compared to all tests after *in vitro* digestion, maintaining a relevant

Table 1
Antioxidant activity of organic coffee husk before and during digestion (μmol EqT/g), and antioxidant capacity remaining (%).

Analysis	Antioxidant activity			
	Coffee Husk	**Gastric phase**	**Intestinal phase Bioaccessible**	**ACR (%)**
DPPH μmol EqT/g	1995.5 ± 1.84^{A}	1171.2 ± 4.1^{B}	1139.0 ± 4.00^{B}	57.07*
FRAP μmol EqT/g	2043.1 ± 8.21^{A}	1931.66 ± 3.27^{B}	1883.98 ± 2.75^{C}	92.21**
ORAC μM EqT/g	65.44 ± 3.12^{A}	40.78 ± 0.65^{B}	33.48 ± 0.19^{C}	51.16***

A – C: Different superscript capital letters in the same row for the same method denote differences ($p \leq 0.05$), based on Tukey's test; */**/*** in the antioxidant capacity remaining (ACR) of the fluids (column).

global antioxidant effect, with the most significant results compared to FRAP (92.21 %, RAC) (Table 1). These results show that CHE is able to maintain its antioxidant potential *in vitro*, although it is affected by the digestive conditions in the gastrointestinal tract. This is probably due to the complexity of the matrix, which provides the digestive process with a range of compounds that can be metabolized in the extract, delaying the degradation of compounds such as phenols, which are responsible for an important part of the antioxidant potential.

This result is considered beneficial for human health, as the phenolic compounds released and preserved from CH can exert antioxidant effects in the digestive tract and help maintain the integrity of the gut (Shahidi et al., 2019).

3.3. Effects of CH on fecal bacterial populations during in vitro fecal fermentation

The relative abundance of *Lactobacillus* spp./ *Enterococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp./ *Prevotella* spp. and *Clostridium histolyticum* in the CH fermentation medium and in the negative control (NC; without fermentable substrate) during 48 h *in vitro* fecal fermentation is shown in Fig. 2. The medium with CH promoted important changes in the relative abundance of microbial groups. It was observed that the abundance of *Lactobacillus* spp./*Enterococcus* spp. and *Bifidobacterium* spp. populations increased significantly ($p \leq 0.05$) during the 48 h of *in vitro* fecal fermentation, with the last group showing a more pronounced proportion.

This result indicates a resistance of fermentable CH components to *in vitro* digestion, which, when made available during *in vitro* fecal fermentation, can be used as substrates by fecal microorganisms. In addition to phenolic compounds, CH is also rich in dietary fiber, and both are components with great potential for metabolization by the human gut microbiota (de Oliveira et al., 2023). Similar results to this research were observed by Massa et al. (2022), who found an increase in the relative abundance of *Lactobacillus* spp./*Enterococcus* spp. and *Bifidobacterium* spp. is considered important for the host, as these microbial groups are generally associated with health-promoting actions that promote beneficial effects at intestinal and systemic levels (da Silva et al, 2023) and therefore may be positive for individuals with type 2 diabetes.

In addition, these results can also be considered relevant as *Lactobacillus* spp./*Enterococcus* spp. and *Bifidobacterium* spp. are the main microbial markers to demonstrate the potential prebiotic effect of a compound. A compound labeled as a prebiotic can have a positive impact on the host's gut and systemic health by positively modulating

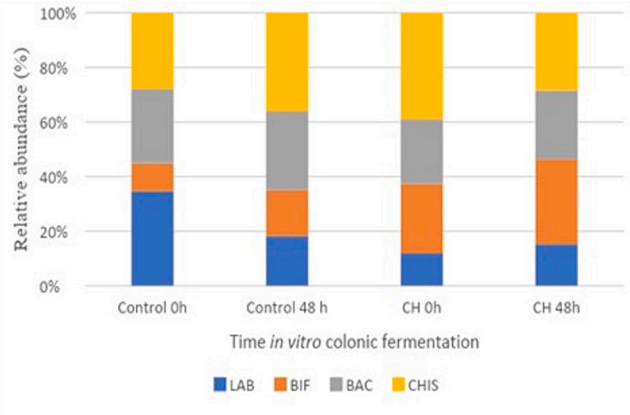

Fig. 2. Relative abundance of different bacterial groups (% average ± standard deviation, n = 3) in media with digested organic coffee husk bran (CH) and negative control (NC; without fermentable substrate) at time zero (baseline), and 48 h of colonic fermentation. Lab 158 specific to *Lactobacillus* spp./ *Enterococcus* spp.; Bif 164 specific to *Bifidobacterium* spp.; Bac 303 specific to *Bacteroides* spp./*Prevotella* spp., and Chis 150 specific to *C. histolyticum*.

the gut microbiota, which becomes even more important when considering the bacterial populations in a diabetic's microbiota (Alarcón Yempén et al., 2021; Gibson et al., 2017). The increase in the relative abundance of *Lactobacillus* spp./*Enterococcus* spp. and *Bifidobacterium* spp. during the fermentation of CH in the fecal material of diabetic patients also proves to be fundamental, as these microbial groups may contribute to the reduction of fasting and postprandial blood glucose levels in diabetic patients through the production of metabolites that act as modulators of enzymes and various hormones (Das & Gnanasambandan, 2023). The results presented in Fig. 2 also show that in the fermentation medium corresponding to CN, there was a significant decrease ($p \leq 0.05$) in the relative abundance of *Lactobacillus* spp./ *Enterococcus* spp. and an increase ($p \leq 0.05$) in the abundance of *Bifidobacterium* spp. after 48 h of *in vitro* fecal fermentation. It is important to emphasize that the percentage of the latter group was lower than that observed in the medium with CH after 48 h of *in vitro* fermentation.

These results are related to the absence of fermentative material in the NC, which prevents a significant increase in the relative abundance of microbial groups and thus differs from what was observed in the medium with CH. The relative abundance of *Bacteroides* spp./*Prevotella* spp. increased ($p \leq 0.05$) in the medium with CH and in NC after 48 h of *in vitro* fecal fermentation (Fig. 2). People with diabetes show differences in the composition of the gut microbiota compared to healthy people (Alarcón Yempén et al, 2021), and according to Gurung et al. (2020), there is a correlation between type 2 diabetics and a significant abundance of this group of microorganisms in their gut microbiota, which could be one of the explanations for the apparent abundance of *Bacteroides* spp./*Prevotella* spp. in both fermentation media, as the feces used in both systems come from diabetics.

In addition, the population of *Bacteroides* spp./*Prevotella* spp. is influenced by the substrates available for fermentation, as they are able to metabolize a variety of components and produce important secondary metabolites such as propionic acid (de Albuquerque et al., 2021). In this way, it can be assumed that the availability of compounds such as phenols and fibers from CH in the fermentation medium led to an increase in the proportion of this microbial group. The relative abundance of C. *histolyticum* increased after 48 h of *in vitro* fermentation in NC ($p \leq 0.05$). On the other hand, an opposite behavior was observed in the medium with CH, leading to a decrease of *C. histolyticum* during 48 h of fecal fermentation *in vitro*. These results suggest that CH component content causes selective modulation of fecal microbiota, which is positive because individuals with type 2 diabetes have genes that favor the growth of opportunistic pathogens (Hameed et al, 2020), and C. *histolyticum* is a group of microorganisms that, when imbalanced in the gut microbiota, can favor intestinal diseases through the production of harmful metabolites, so it is desirable for the health of the host to reduce their abundance (de Medeiros et al., 2021).

Massa et al. (2022) also observed a reduction in the abundance of C. *histolyticum* during *in vitro* fecal fermentation in a medium containing Jabuticaba by-products and strongly associated this result with the presence of fibers from the by-product as well as phenolic compounds that have an inhibitory effect on the *C. histolyticum* population. These findings support the results observed in this study, as CH is rich in fibers and phenolic compounds, as previously mentioned.

3.4. Metabolism of phenolic compounds in organic coffee husks (CH)

During *in vitro* digestion and fermentation of the CH investigated in this study, the metabolism of the phenolic compounds of the matrix was monitored and a Pearson correlation analysis was used to investigate the dynamics of the compounds during gastrointestinal digestion and *in vitro* fermentation in the feces. The results of the correlation analysis were presented in the form of a hierarchical cluster and heat map (Fig. 3 and Table S1), which categorizes the studied samples into 4 groups based on the expression of 17 phenolic compounds identified during the *in vitro* tests.

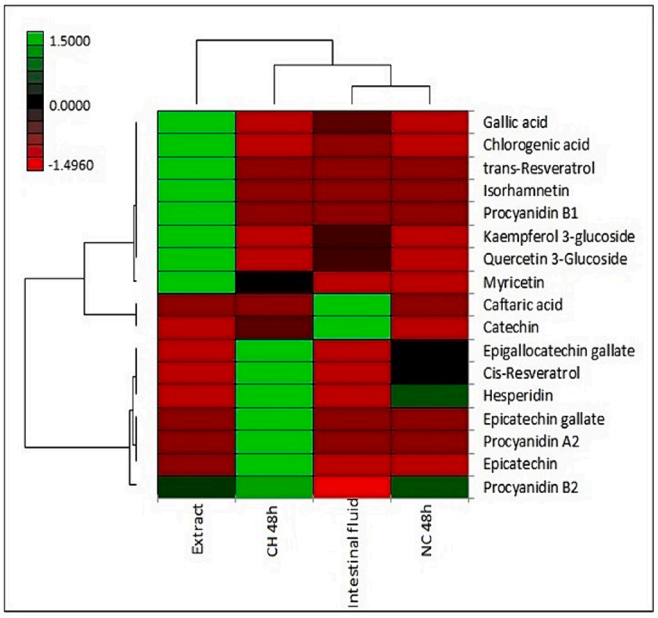

Fig. 3. Map of the Pearson's correlation for phenolic compound profile variable data measured in digested organic coffee husk medium during 48 h of *in vitro* colonic fermentation. Intestinal fluid corresponds to zero fermentation time. NC 48 h represents the negative control (fermentation medium without coffee husk), CH 48 h the intestinal fluid digested with coffee husk and extract of organic coffee husk.

The first group is represented by the CH extract before *in vitro* digestion, which was evaluated to verify the compounds most present in its composition. In Fig. 3, it can be seen that among the 17 compounds identified, chlorogenic acid, recognized as the most abundant compound in coffee husks (Marques et al., 2022), has the highest expression together with gallic acid, *trans*-resveratrol, isorhmnetin, procyanidin B1, kaempferol-3-glucoside, quercetin-3-glucoside and myricetin.

The hierarchical group represented by the intestinal fluid (Fig. 3) reflects the bioaccessible phenolic compounds after *in vitro* digestion of the CH extract. Compared to the undigested extract, a stronger expression is observed for a lower number of compounds, with caftaric acid and catechin showing the highest concentrations. Silva et al (2024) explained that this effect is due to the chemical and enzymatic transformations that the phenolic compounds undergo during digestion, potentially altering the profile and content of bioavailable phenolic compounds.

Caftaric acid, a derivative of the combination of caffeic acid and tartaric acid, shows an increased concentration after digestion, probably related to the degradation of chlorogenic acid, which was particularly present in the extract before digestion. Remarkably, chlorogenic acid is composed of caffeic acid monomers, suggesting a plausible release of this compound during digestion and its probable conversion to caftaric acid. Conversely, catechin, a component of procyanidins, may have been released by the metabolism of procyanidin B1, which had higher concentrations in the extract prior to digestion (Mcdade et al., 2020; Silva et al., 2024; Zheng et al., 2020).

The designations "CH 48 h" and "NC 48 h" describe the phenolic profiles detected in the media with CH and the negative control (NC) in an *in vitro* fecal fermentation (Fig. 3). It is evident that after 48 h of *in vitro* fecal fermentation, a considerable number of compounds were expressed in the CH medium, in contrast to the limited expression observed in the NC.

In CH medium, compounds such as epigallocatechin gallate, *cis*-resveratrol, hesperidin, epicatechin gallate, procyanidin A2, procyanidin B2 and epicatechin showed higher levels, although they were less expressed in CH both before and after *in vitro* digestion (see Fig. 3). Pérez-Burillo et al. (2019) observed analogous trends in phenolic compounds in green and roasted coffee after *in vitro* fecal fermentation, suggesting the metabolic capabilities of gut microorganisms in processing these compounds yielding different phenolic products. This microbial activity explains the observed expression of compounds in the CH medium and highlights the utilization of CH compounds as substrates by the fecal microbiota.

Populations of the gut microbiota have the ability to metabolize phenolic compounds via specific enzymes, producing derivatives that are used as carbon sources by the gut microbial community (Gonthier et al., 2006; Massa et al., 2022). Consumption of available phenolic compounds can selectively increase the abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. microorganisms known to produce short-chain fatty acids a phenomenon that has been associated with improved lipid metabolism and consequent beneficial effects on glucose metabolism in people with type 2 diabetes (da Silva et al., 2023).

The initial pool of phenolic compounds available for fermentation by the gut microbiota is metabolized, producing phenols that can yield other compounds, including catechin, which is derived from phenols of the procyanidin class. The term 'procyanidins' refers to the monomers of catechin as procyanidins B, while the monomers of epicatechin connected by ester and ester-ester bonds are referred to as procyanidins A. Some of the procyanidin isomers include epicatechin, catechin, epicatechin-3-catechin, catechin-3-epicatechin, and epicatechin-3-(2-catechin) (Mcdade et al., 2020; Smeriglio et al, 2017). The maintenance of phenolic compounds in the intestinal environment is not only important for increasing the relative abundance of beneficial microorganisms such as *Lactobacillus* spp. and *Bifidobacterium* spp. but also for curbing the proliferation of non-beneficial microorganisms such as *C. histolyticum* (da Silva et al., 2023), as previous research has shown, suggesting a possible prebiotic effect of CH.

In addition, the symbiotic interaction between microorganisms and phenolic compounds during intestinal fermentation provides multiple benefits for host health. First of all, the metabolized phenolic compounds positively influence the proliferation, viability and function of beneficial microorganisms in the gut. In addition, their consumption leads to more active phenolic derivatives or other by-products that bring additional benefits to host health (de Medeiros et al., 2021). This underlines the paramount importance of these compounds, especially for people suffering from diseases such as type 2 diabetes.

4. Conclusion

The results of this study indicate that organic coffee husks are a safe resource for human consumption. This is evidenced by the non-toxicity of CHE and its ability to maintain cell viability and protect Caco-2 cells. This maintenance of cell viability and protection is likely due to the bioactive properties of CH, which showed remarkable antioxidant capabilities in DPPH, FRAP and ORAC assays and retained this potential after digestion in the gastrointestinal tract *in vitro*. In addition, CH showed the ability to positively modulate the intestinal microbiota of individuals with type 2 diabetes, as evidenced by an increase in the amount of beneficial gut microorganisms and a simultaneous decrease in the amount of non-beneficial microorganisms during *in vitro* fecal fermentation. These results suggest a potential prebiotic effect of CH, in which it provides substrates such as fibers and phenolic compounds for selective metabolism by microorganisms in the fecal microbiota and provides by-products that are beneficial for host health.

In summary, CH has properties that should be explored beyond mere waste utilization. Through its antioxidant properties, protection of cells and promotion of the proliferation of beneficial gut microorganisms, CH has the potential to make an important contribution to the promotion of human health, especially in people suffering from type 2 diabetes. This study represents just the beginning; it underscores the necessity for further investigation in subsequent research projects aimed at deepening our understanding of the potential of coffee husks.

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CRediT authorship contribution statement

Thaianaly Leite Abreu: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Gezaildo Santos Silva:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Antonia Dayane Jenyffer de Farias Marques:** Formal analysis, Data curation. **Juliana Santos de Espindola:** Writing – review & editing, Visualization, Methodology, Data curation. **Bianca Beatriz Torres de Assis:** Formal analysis, Data curation. **Karoliny Brito Sampaio:** Formal analysis, Data curation. **Thatyane Mariano Rodrigues de Albuquerque:** Writing – review & editing, Formal analysis, Data curation. **Maria Teresa Bertoldo Pacheco:** Writing – review & editing, Data curation, Conceptualization. **Fabiana Andrea Barrera Galland:** Writing – review & editing, Conceptualization. **Evandro Leite de Souza:** Writing – review & editing, Conceptualization. **Marciane Magnani:** Writing – review & editing, Conceptualization. **Marta Suely Madruga:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Taliana Kênia Alencar Bezerra:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

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.

Data availability

No data was used for the research described in the article.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114730.

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