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EFFECTS OF *IN VITRO* DIGESTION AND *IN VITRO* COLONIC FERMENTATION ON STABILITY AND FUNCTIONAL PROPERTIES OF YERBA MATE (*Ilex paraguariensis* A. St. Hil.) BEVERAGES

VANESA GESSER CORREA

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Dissertação apresentada ao programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de mestre em Ciência de Alimentos.

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Orientadora
Profª Drª Rosane Marina Peralta

BIOGRAFIA

Vanesa Gesser Correa nasceu em 12/03/1992 na cidade de Salto do Lontra-PR.

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Tem experiência na área de bioquímica de alimentos, atuando principalmente nos seguintes temas: alimentos funcionais e antioxidantes.

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Além do artigo principal que compõe sua dissertação de mestrado, é co-autora dos seguintes artigos científicos.

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Dedico

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APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico:

1 Autores: Vanesa G. Correa, Geferson A. Gonçalves, Anacharis B. de Sá-Nakanishi, Isabel C. F. R. Ferreira, Lillian Barros, Maria I. Dias, Eloá A. Koehnlein, Adelar Bracht e Rosane M. Peralta.

Artigo: Effects of *in vitro* digestion and *in vitro* colonic fermentation on stability and functional properties of yerba mate (*Ilex paraguariensis* A. St. Hil.) beverages.

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GENERAL ABSTRACT

INTRODUCTION AND AIMS: The yerba mate or mate (YM) (*Ilex paraguariensis* A. St. Hil.) is a plant native from Paraguay, Uruguay, Argentina and Brazil. The powder of YM leaves and thin stems is used for the preparation of several stimulant drinks. The three most important are chimarrão (hot water extract of green dried leaves), tererê (cold water extract of green dried leaves) and mate tea (hot water extract of toasted leaves). Yerba mate is known to be rich in phenolic acids such as caffeic acid and chlorogenic acid and their derivatives and flavan-3-ols. Due this, their consumption has been considered beneficial for health and different bioactive properties have been related. It is well known that flavonoids and phenolic acids are extensively metabolized after ingestion and gastrointestinal absorption, being usually transformed into plasma metabolites with lower antioxidant activity than the precursor molecules. Studies mimetizing the digestion process have shown that the content of bioactive compounds is modified when passing through the various compartments of the gastrointestinal tract in consequence of pH alterations, enzymes action, and the metabolic activity of the intestinal microbiota. The aim of this work was to mimic the gastrointestinal digestion and the colonic fermentation of chimarrão, tererê and mate tea in order to get a possible estimate of the bioactive compounds from each preparation that effectively reach the circulation and the tissues.

MATERIAL AND METHODS: Raw and toasted yerba mate were obtained from reliable commercial sources in the South of Brazil. The beverages were prepared in the way they are popularly consumed. For the preparation of chimarrão and tererê, 1.5 L of water was added at 80 °C and 10ºC, respectively to 85 g of raw (green) yerba mate. After 5 min, the mixtures were filtered in a vacuum pump. For mate tea preparation, 1.5 L of water at 90 ºC was added to 85 g of toasted yerba mate. After 5 min, the mixtures were also filtered in a vacuum pump. The three extracts were lyophilized and kept at -20 °C until analysis. *In vitro* gastrointestinal digestion was carried out simulating the oral, gastric and small intestine phases. For *in vitro* colonic fermentation a carbonate-phosphate buffer was used as the fermentation medium and the inoculum was prepared from fresh feces collected from male *Wistar* rats fed with standard diets and that had not received antibiotics at any time. The phenolic compounds were analyzed by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). For the evaluation of antioxidant activity, six different methods were used: FRAP, ORAC, DPPH, ABTS, TBARS assay and inhibition of mitochondrial ROS production. To screen the antibacterial activity of the lyophilized extract seven Gram-negative bacteria and five Gram-positive bacteria were used. MIC determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay. MIC was defined as the lowest extract concentration that prevented changes in method and exhibited inhibition of bacterial growth. Sulforhodamine B assay was performed for cytotoxicity analysis. Four human tumor cell lines were tested: MCF-7 (breast adenocarcinoma), NCIH460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). For evaluation of the cytotoxicity in non-tumor cells, a cell culture (assigned as PLP2) was prepared from a freshly harvested porcine liver. The results were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* Student–Newman–Keuls testing. P values <0.05 were considered to be significant. The error parameters presented in tables are standard errors of the means. This treatment was carried out using the GraphPad Prism software (version 5.0).

RESULTS AND DISCUSSION: Chimarrão presented the highest level of total phenolic compounds and flavonoids (111.46 ± 3.85 mg/g extract and 5.61±0.06 mg/g extract, respectively), followed by tererê (69.01 ± 4.72 and 1.00 ± 0.01 mg/g extract, respectively), and

mate tea (64.35 ± 0.73 and 0.02 ± 0.01 mg/g extract, respectively). The lowest amount of phenolic compounds in mate tea can be explained by the possible degradation of some compounds by the high temperatures applied in the toasting process. After *in vitro* digestion total phenolic compounds of chimarrão, tererê and mate tea decreased by 74.69±5.48, 69.01±4.72 and 51.60±1.89 mg/g extract, respectively, representing reductions of 33%, 24% and 20%, respectively. This behaviour indicates that the transformation of the phenolic compounds may be influenced by pH changes and by interactions with other constituents during *in vitro* digestion. After colonic fermentation, no significant alterations in the total phenolic compounds were observed in chimarrão and tererê, while in mate tea, total phenolic compounds decreased by 34.64± 0.20 mg/g extract, what represents a reduction of 33%. In general, the *in vitro* gastrointestinal and colonic fermentation caused a reduction, to a greater or lesser degree, in the antioxidant capabilities of the yerba mate beverages, except in the ABTS assay. Although the decreases in the antioxidant activities were statistically significant ($p \leq 0.05$) in several cases, the extracts maintained antioxidant properties. The green and toasted yerba mate extracts exhibited antibacterial activity against all Gram positive and Gram negative bacteria tested. Also, all yerba mate extracts were more active against Gram positive bacteria, especially *Staphylococcus aureus*, MRSA-methicillin-resistant *Staphylococcus aureus*, and MSSA-methicillin-susceptible Staphylococcus aureus. In general, the *in vitro* digestion and colonic fermentation barely affected the antimicrobial activities of the extracts. However, after *in vitro* digestion and colonic fermentation, the extracts were more active against *S. aureus*, MRSA and MSSA. The crude extracts showed cytotoxicity against HeLa cells. This cytotoxicity was slightly affected by *in vitro* digestion and colonic fermentation. Interestingly, the colonic fermentation improved the cytotoxicity of the mate tea extract against all tumor cell lines, except HepG2. None of the tested extracts showed toxicity against normal (non-tumor) porcine liver primary cells (GI_{50}>400 µg/mL).

CONCLUSIONS: The results of this study demonstrate, for the first time, the effects of both *in vitro* digestion and *in vitro* colonic fermentation of yerba mate prepared in the three most common forms of consumption (chimarrão, tererê and mate tea). Despite the decrease in the phytochemicals content, yerba mate beverages maintained their functional properties such as antioxidant, antibacterial and antitumor activities after *in vitro* gastrointestinal digestion and *in vitro* colonic fermentation.

Key words: chlorogenic acid, colonic fermentation, *Ilex paraguariensis*, *in vitro* gastrointestinal digestion, yerba mate.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS: A erva-mate ou mate (*Ilex paraguariensis* A. St. Hil.) é uma planta nativa do Paraguai, Uruguai, Argentina e Brasil. O pó das folhas da planta e hastes finas é usado para a preparação de várias bebidas estimulantes, os três mais importantes são o chimarrão (extrato de água quente de folhas verdes secas), o tererê (extrato de água fria de folhas verdes secas) e o chá mate (extrato de água quente de folhas torradas). *I. paraguariensis* é conhecido por ser rico em ácidos fenólicos, tais como ácido cafeico e ácido clorogênico e seus derivados e flavan-3-ols. Devido a isso, seu consumo tem sido considerado benéfico para a saúde e diferentes propriedades bioativas foram relacionadas à planta. É bem conhecido que os flavonoides e os ácidos fenólicos são extensamente metabolizados após ingestão e absorção gastrointestinal, sendo normalmente transformados em metabólitos plasmáticos com menor atividade antioxidante do que as moléculas precursoras. Estudos que mimetizam o processo de digestão mostraram que o conteúdo de compostos bioativos é modificado quando se passa pelos vários compartimentos do trato gastrointestinal em decorrência de alterações de pH, ação de enzimas e atividade metabólica da microbiota intestinal. O objetivo deste trabalho foi mimetizar a digestão gastrointestinal e a fermentação colônica do chimarrão, tererê e chá mate, a fim de obter uma possível estimativa dos compostos bioativos de cada preparação que efetivamente atingem a circulação e os tecidos.

MATERIAL E MÉTODOS: A erva-mate verde e tostada foi obtida de fontes comerciais no Sul do Brasil. As bebidas foram preparadas da forma como são consumidas popularmente. Para a preparação de chimarrão e tererê, 1,5 L de água foram adicionados a 80 °C e 10 °C, respectivamente, a 85g de erva-mate verde. Após 5 min, as misturas foram filtradas numa bomba de vácuo. Para a preparação de chá mate, adicionou-se 1,5 L de água a 90 °C a 85g de erva-mate torrada. Após 5 min, a mistura foi também filtrada numa bomba de vácuo. Os três extratos foram liofilizados e mantidos a -20 °C até à análise. A digestão gastrointestinal *in vitro* foi realizada simulando as fases oral, gástrica e do intestino delgado. Para a fermentação colônica *in vitro* utilizou-se um tampão carbonato-fosfato como meio de fermentação e o inoculo foi preparado a partir de fezes frescas recolhidas de ratos *Wistar* machos, alimentados com dietas padrão e que não tinham recebido antibióticos em qualquer momento. Os compostos fenólicos foram quantificados por Cromatografia Líquida acoplada à Espectrometria de Massa (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, EUA). Para a avaliação da atividade antioxidante, foram utilizados seis métodos diferentes: FRAP, ORAC, DPPH, ABTS, ensaio TBARS e inibição da produção de ROS mitocondrial. Para pesquisar a atividade antibacteriana do extrato liofilizado foram utilizadas sete bactérias Gram-negativas e cinco bactérias Gram-positivas. As determinações da Concentração Inibitória Mínima (CIM) foram realizadas pelo método de microdiluição e pelo ensaio colorimétrico rápido de cloreto de p-iodonitrotetrazólio (INT). A CIM foi definida como a concentração mais baixa de extrato que impede alterações no método e exibiu inibição do crescimento bacteriano. O ensaio de sulforodamina B foi realizado para análise de citotoxicidade. Foram testadas quatro linhas celulares de tumor humano: MCF-7 (adenocarcinoma da mama), NCIH460 (câncer de pulmão de não pequenas células), HeLa (carcinoma cervical) e HepG2 (carcinoma hepatocelular). Para a avaliação da citotoxicidade em células não tumorais, preparou-se uma cultura de células (designada como PLP2) a partir de fígado de suíno. Os resultados foram analisados utilizando a análise de variância unidirecional (ANOVA) seguida de teste post hoc Student-Newman-Keuls. P valores $<0,05$ foram considerados significativos. Os parâmetros de erro apresentados nas tabelas são erros padrão dos meios. Este tratamento foi realizado utilizando o software GraphPad Prism (versão 5.0).

RESULTADOS E DISCUSSÃO: Chimarrão apresentou o maior nível de compostos fenólicos totais e flavonoides (111,46 ± 3,85 mg/g de extrato e 5,61 ± 0,06 mg/g de extrato, respectivamente), seguido de tererê (69,01 ± 4,72 e 1,00 ± 0,01 mg/g de extrato, respectivamente) e chá mate (64,35 ± 0,73 e 0,02 ± 0,01 mg/g de extrato, respectivamente). A menor quantidade de compostos fenólicos no chá mate pode ser explicada pela possível degradação de alguns compostos pelas altas temperaturas necessárias no processo de tostar. Após a digestão *in vitro*, os compostos fenólicos totais de chimarrão, tererê e chá mate diminuíram para 74,69 ± 5,48, 69,01 ± 4,72 e 51,60 ± 1,89 mg/g de extrato, respectivamente, representando reduções de 33%, 24% e 20%, respectivamente. Este comportamento indica que a transformação dos compostos fenólicos pode ser influenciada por alterações de pH e por interações com outros constituintes durante a digestão. Após a fermentação colônica, não foram observadas alterações significativas nos compostos fenólicos totais no chimarrão e no tererê, enquanto que no chá mate houve diminuição em 34,64 ± 0,20 mg/g de extrato, o que representa uma redução de 33%. De um modo geral, a digestão gastrointestinal e fermentação colônica provocaram uma redução, em maior ou menor grau, das capacidades antioxidantes das bebidas de erva-mate, exceto no ensaio ABTS. Embora as diminuições nas atividades antioxidantes tenham sido estatisticamente significativas ($p \leq 0,05$) em vários casos, os extratos mantiveram propriedades antioxidantes. Os extratos de erva-mate verde e torrada exibiram atividade antibacteriana contra todas as bactérias, Gram positivas e Gram negativas, testadas. Além disso, todos os extratos foram mais ativos contra bactérias Gram positivas, especialmente *Staphylococcus aureus, Staphylococcus aureus MRSA* resistente à meticilina e *Staphylococcus aureus MSSA* sensível à meticilina. Em geral, a digestão *in vitro* e a fermentação colônica pouco afetaram as atividades antimicrobianas dos extratos. Contudo, após a digestão e fermentação colônica, os extratos foram mais ativos contra *S. aureus*, MRSA e MSSA. Os extratos brutos mostraram citotoxicidade contra células HeLa. Esta citotoxicidade foi ligeiramente afetada pelas etapas da digestão. Curiosamente, a fermentação colônica melhorou a citotoxicidade do extrato de chá mate contra todas as linhas celulares tumorais testadas, exceto HepG2. Nenhum dos extratos testados apresentou toxicidade contra células primárias de fígado de porco normal (não tumorais) ($GI_{50} > 400$ μg/mL).

CONCLUSÕES: Os resultados deste estudo demonstram, pela primeira vez, os efeitos da digestão *in vitro* e da fermentação colônica de erva-mate preparada nas três formas de consumo mais comuns (chimarrão, tererê e chá mate). Apesar da diminuição do teor de fitoquímicos, as bebidas mantiveram suas propriedades funcionais como atividades antioxidantes, antibacterianas e antitumorais após as fases da digestão mimetizadas.

Palavras chaves: ácido clorogênico, fermentação colônica, *Ilex paraguariensis*, digestão gastrointestinal *in vitro*, erva-mate.

ARTICLE

Effects of *in vitro* digestion and *in vitro* colonic fermentation on stability and functional properties of yerba mate (*Ilex paraguariensis* A. St. Hil.) beverages

Running title: Stability and functional properties of yerba mate beverages

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Abstract. Yerba mate (*Ilex paraguariensis*) is a native plant from South America from which different beverages (chimarrão, tererê and tea mate) with high bioactive contents are obtained. The aim of this study was to evaluate the influence of *in vitro* gastrointestinal digestion and colonic fermentation on the stability of the polyphenols and on the antioxidant, antimicrobial and antitumoral activities of the yerba mate beverages. LC-DAD-ESI/MSn analysis revealed that both the *in vitro* digestion and the colonic fermentation caused a pronounced decrease in 3,5-*O*-dicaffeoylquinic acid and 5-*O*-caffeoylquinic acid in the preparations. However, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid and salvianolic acid I, were only barely affected in all preparations. Despite the decrease in the phytochemicals content, yerba mate beverages maintained their functional properties such as antioxidant, antibacterial and antitumoral activities.

Keywords: antioxidant activity; antibacterial activity; antitumoral activity; chlorogenic acid; colonic fermentation; *Ilex paraguariensis*; *in vitro* gastrointestinal digestion; yerba mate.

1. Introduction

The yerba mate or mate (YM) (*Ilex paraguariensis* A. St. Hil.) is a plant native from Paraguay, Uruguay, Argentina and Brazil. The powder of YM leaves and thin stems is used for the preparation of several stimulant drinks. The three most important are *chimarrão* (hot water extract of green dried leaves), *tererê* (cold water extract of green dried leaves) and *mate tea* (hot water extract of toasted leaves) (Bracesco, Sanchez, Contreras, Menini, & Gugliucci, 2011; Lima, de Oliveira, da Silva, Maia, de Moura, & Lisboa, 2014a). The Portuguese word *chimarrão* designates the preparation that in Spanish speaking countries is usually designated by the word *mate.*

The consumption of *chimarrão*, *tererê* and *mate tea* is high in countries where *I. paraguariensis* is cultivated: the yerba consumption reaches 8-10 kg per person per year in Uruguay, 6.5 in Argentina and 3-5 in Southern Brazil (Cardozo Junior & Morand, 2016). In countries from North America, Europe and Asia the toasted leaves of the plant are used for the production of teas and energetic drinks (Cardozo Junior & Morand, 2016).

Consumption of yerba mate has been considered beneficial to health (Bracesco et al., 2011). Yerba mate is used for improving lipid profiles and blood circulation (Lima et al., 2014b, Kim, Oh, Kim, Chae & Chae, 2015). It is also used as diuretic and antirheumatic (Isolabella, Cogoi, López, Anesini, Ferraro, & Filip., 2010), as well as antioxidant (Souza et al., 2015). Cytotoxic and antiproliferative activities against cancer cells as well as anti-inflammatory, hepatoprotective, neuroprotective and anti-depressant effects have also been ascribed to yerba mate (de Mejía, Song, Heck, & Ramírez-Mares, 2010; Heck & de Mejia, 2007; Lima et al., 2014a).

Yerba mate is known to be rich in phenolic acids such as caffeic acid and chlorogenic acid and their derivatives and flavan-3-ols, such as (+)-catechin (Bracesco et al., 2011; da Silveira, Meinhart, de Souza, Teixeira Filho & Godoy, 2016; Souza et al., 2015). Other

compounds frequently found in the extracts are: gallic, syringic, ferulic, *p*-coumaric acids, rutin, methylxanthines (caffeine and theobromine), saponins and tannins (Bracesco et al., 2011; da Silveira et al., 2016; de Mejía et al., 2010; Murakami et al., 2013).

It is well known that flavonoids and phenolic acids are extensively metabolized after ingestion and gastrointestinal absorption, being usually transformed into plasma metabolites with lower antioxidant activity than the precursor molecules. Studies mimetizing the digestion process have shown that the content of bioactive compounds is modified when passing through the various compartments of the gastrointestinal tract in consequence of pH alterations, enzymes action, and the metabolic activity of the intestinal microbiota (Boaventura et al., 2015; Correa-Betanzo, Allen-Vercoe, McDonald, Schroeter, Corredig & Paliyath, 2014). Time and temperature of digestion can also further influence the final outcome in both qualitative and quantitative terms. For example, about one-third of the chlorogenic acid content is absorbed in the small intestine, while two-thirds reach the colon where they can be transformed by the microbiota (Correa-Betanzo et al., 2014; Stalmach, Steiling, Williamson & Crozier, 2010). Taking into account these notions, the aim of this study was to mimic the gastrointestinal digestion and the colonic fermentation of chimarrão, tererê and mate tea in order to get a possible estimate of the bioactive compounds from each preparation that effectively reach the circulation and the tissues. Besides quantifying the compounds after the gastrointestinal digestion and the colonic fermentation, an evaluation of the resulting antioxidant, antitumoral and antibacterial activities was also performed.

2. Materials and methods

2.1. Standards and Reagents

Salivary alpha-amylase, pancreatin, pepsin, bile extract, gallic acid, catechin, 2,2-azinobis (3-ethyl benothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-azobis (2-amidinopropane) dihydrochloride (AAPH), formic acid lipopolysaccharide (LPS), dexamethasone, sulforhodamine B, trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma-Aldrich Co (St Louis, MO, USA). Acetonitrile from Fisher Scientific (Lisbon, Portugal) was of HPLC grade (99.9%). Phenolic standards were from Extrasynthèse (Genay, France). The Griess Reagent System Kit was purchased from Promega (Madison, WI, USA). Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), foetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) were purchased from Hyclone (Logan, UT, USA). All other general laboratory reagents were of analytical grade and purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Sample preparation

Raw and toasted yerba mate were obtained from reliable commercial sources and producers in the South of Brazil. The beverages were prepared in the way they are popularly consumed. For the preparation of chimarrão and tererê, 1.5 L of water was added at 80 °C and 10 ^{o}C, respectively to 85 g of raw (green) yerba mate. After 5 min, the mixtures were filtered in a vacuum pump. For mate tea preparation, 1.5 L of water at 90 ^{o}C was added to 85 g of

toasted yerba mate. After 5 min, the mixture was also filtered in a vacuum pump. The three extracts were lyophilized and kept at -20 °C until analysis.

2.2. In vitro digestion

In vitro gastrointestinal digestion was simulated as previously described (Koehnlein et al., 2016). Briefly, 13 g of lyophilized extract was mixed with 39 mL of artificial saliva solution (2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, 8 g NaCl in 1 L of distilled water). The pH was adjusted to 6.75, at the temperature of 37 °C and α-amylase was added to obtain 200 U of enzyme activity. This mixture was shaken at 150 rpm for 10 min. After this time the pH was adjusted to 1.2 by the addition of 5 mol/L HCl and 39 mL of artificial gastric fluid (0.32 g pepsin in 100 mL of 0.03 M NaCl, pH 1.2) was added. The mixture was incubated at 37 °C for 120 min, on a shaker with an agitation of 150 rpm. Finally, the pH was adjusted again to 6.0 with $NaHCO_3$ following the addition of 6.5 mL of NaCl (120 mM), 6.5 mL of KCl (5 mM) and 39 mL of artificial intestinal fluid (0.15 g of pancreatin and 0.9 g of bile extract in 100 mL of 0.1 M $NaHCO_3$). The mixture was incubated at 37 °C for 60 min, at 150 rpm. Thereafter the samples were lyophilized and kept at -20°C.

2.3. In vitro colonic fermentation

The fermentation medium was a carbonate-phosphate buffer and it was prepared as previously described (Karppinen, Liukkonen, Aura, Forssell & Poutanen, 2000) with modifications. The mineral medium was adjusted to pH 7.0 and glucose was added to a final concentration of 0.8%. The mixture was purged with nitrogen until the anaerobic indicator (methylene blue) became colorless

The inoculum was prepared from fresh feces collected from male *Wistar* rats fed with standard diets and that had not received antibiotics at any time. Immediately after collecting, the material was homogenized with the culture medium and samples at a ratio of 1:10 (w/v).

The bottles were bubbled again with nitrogen for the same time as the previous one and sealed airtight. Afterwards, they were incubated at 37 ºC for 24 h with shaking at 50 rpm, aiming to simulate the condition in the colonic lumen. A control with the culture medium and inoculum was prepared. Thereafter, the material was submitted to ultra-centrifugation at 31,000 rpm for 30 min, sterilized by filtration, and lyophilized.

2.4. Analysis of phenolic compounds

The lyophilized extracts were re-dissolved in water and analyzed by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) (Bessada, Barreira, Barros, Ferreira, & Oliveira, 2016). For the double online detection 280 and 370 nm were used as the preferred wavelengths for DAD. The mass spectrometer (MS) was connected to the HPLC system via the DAD cell outlet. The MS detection was performed in the negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. The identification of the phenolic compounds was performed using standard compounds, when available, by comparison of their retention times, UV-vis and mass spectra; and also, by comparing the obtained information with data available in the literature giving a tentative identification. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The results were expressed as mg/g of extract.

2.5. Evaluation of antioxidant activity

Six different methods were used to evaluate the antioxidant activity: FRAP, ORAC, DPPH, ABTS, TBARS assay and inhibition of mitochondrial ROS production. Successive dilutions of the stock solution were made and used for assaying the antioxidant activity of the

160 samples. The sample concentrations (mg/mL) providing 50% of antioxidant activity were
161 calculated from the graphs of antioxidant activity against the sample concentrations. Trolox
162 was used as a positive control.

163 The reduction power of the ferric ion (FRAP) and the oxygen radical absorbance radical
164 (ORAC) were evaluated as previously described (Koehnlein et al., 2016). Standard curves were
165 constructed with trolox (r^2=0.99) and the results were expressed as µmol trolox equivalents
166 (TE)/mg of extract.

167 The DPPH (2,2-diphenyl-1-picrylhydrazyl radical) and ABTS (2,2-azino-bis (3-
168 ethylbenzothiazoline-6-sulphonate cation) assays were conducted as described previously
169 (Correa et al, 2015). The percentage of DPPH and ABTS discoloration were calculated using
170 the following equation: $[(A_{CONTROL}-A_{SAMPLE}) / A_{CONTROL}] \times 100$. The results were expressed as
171 IC_{50} values (sample concentration providing 50% of antioxidant activity).

172 Inhibition of the production of thiobarbituric acid reactive substances (TBARS) was
173 evaluated essentially as described by Correa et al. (2015), except that rat brains instead of
174 porcine brains were used as the lipid source. The color intensity of the malondialdehyde-
175 thiobarbituric acid (MDA-TBA) was measured at the wavelength of 532 nm. The results were
176 calculated as inhibition ratio (%) using the following equation: $[(A_{CONTROL} - A-$
177 $_{SAMPLE})/A_{CONTROL}] \times 100$. The results were expressed as IC_{50} values.

178 Inhibition of the mitochondrial reactive oxygen species production (real time ROS
179 production) was carried out as previously describe (Comar et al., 2013). Firstly, mitochondria
180 were isolated from rat livers. In the following, ROS production, basically H_2O_2, was estimated
181 by measuring the linear fluorescence increase (504 nm for excitation and 529 nm for emission)
182 due to 2'-7'-dichlorofluorescein (DCF) formation from the reduced form of 2'-7'-
183 dichlorofluorescein (DCFH) via oxidation by H_2O_2 in the presence of horseradish peroxidase.
184

2.6. Antibacterial activity evaluation

The lyophilized samples were dissolved in water at a concentration of 10 mg/mL and then submitted to further dilutions. The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal. Seven Gram-negative bacteria (*Escherichia coli*, *E. coli* ESBL (extended spectrum of beta-lactamase), *Klebsiella pneumoniae*, *K. pneumoniae* ESBL, *Morganella morganii*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from urine and expectoration) and five Gram-positive bacteria (*MRSA*- methicillin-resistant *Staphylococcus aureus*, *MSSA*-methicillin-susceptible *Staphylococcus aureus*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis*) were used to screen the antibacterial activity of the lyophilized extract. MIC determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay (Kuete et al., 2011a; Kuete et al., 2011b) with some modifications. MIC was defined as the lowest extract concentration that prevented this change and exhibited inhibition of bacterial growth.

Three negative controls (MHB/TSB, the extract, and medium with antibiotic) and a positive control (MHB and each inoculum) were prepared. For the Gram-negative bacteria, negative control antibiotics, such as amikacin (*K. pneumoniae* ESBL and *P. aeruginosa*), tobramycin (*A. baumannii*), amoxicillin/clavulanic acid (*E. coli* and *K. pneumoniae*) and gentamicin (*E. coli* ESBL) were used. For the Gram-positive bacteria, ampicillin (L. monocytogenes) and vancomycin (MSSA, MRSA and *E. faecalis*) were used as controls. The antibiotic susceptibility profile of Gram negative and Gram positive bacteria has been already described by Dias et al. (2016).

2.7. Evaluation of cytotoxic properties

The lyophilized samples were dissolved in water at 4 mg/mL and then submitted to further dilutions. Four human tumor cell lines were tested: MCF-7 (breast adenocarcinoma), NCIH460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Sulforhodamine B assay was performed according to a procedure previously described (Barros et al., 2013). For evaluation of the cytotoxicity in non-tumor cells, a cell culture (assigned as PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established previously (Abreu et al., 2011). As a positive control ellipticine was used and the results were expressed in GI_{50} values (concentration that inhibited 50% of the net cell growth).

2.8. Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* Student–Newman–Keuls testing. P values <0.05 were considered to be significant. The error parameters presented in tables are standard errors of the means. This treatment was carried out using the GraphPad Prism software (version 5.0).

3. Results and discussion

3.1. Effects of in vitro digestion and colonic fermentation on phenolic compounds of yerba mate beverages

Retention time, wavelengths of maximum absorption in the visible region, mass spectral data and tentative identification of the phenolic compounds present in the three preparations of *I. paraguariensis*, chimarrão, tererê and mate tea, are presented in Table 1. An illustrative HPLC phenolic profile of mate tea crude extract obtained at 280 nm and 370 nm for phenolic acids and flavonoids is presented in Figure S1. Thirteen phenolic compounds were identified,

ten phenolic acids (chlorogenic, caffeic and rosmarinic acids derivatives), and three flavonoids, flavonol derivatives, such as quercetin-3-*O*-rutinoside, kaempherol-3-*O*-rutinoside and isorhametin-3-*O*-rutinoside. From the 13 molecules, eleven (compounds 1 to 6, 8 to 11 and 13) have already been identified by Souza et al. (2015) in a water-methanol extract of green yerba mate. The two additional compounds identified in the herein study were, 1,3-*O*-dicaffeoylquinic acid (compound 7) and isorhamnetin-3-*O*-rutinoside (compound 12), which are present in high amounts in toasted yerba mate, and not in green yerba mate. For this reason, they appear in chimarrão and tererê only in trace values.

Alcoholic and hydro-alcoholic extracts of *I. paraguariensis* leaves have been described as being rich in chlorogenic acid (CGA), a group of compounds comprising hydroxycinnamates, such as caffeic, ferulic and *p*-coumaric acids, linked to quinic acid to form a range of conjugated structures known as caffeoylquinic acids (CQA), feruloylquinic acids (FQA) and *p*-coumaroylquinic acids (*p*-CoQA) (Souza et al., 2015). In the present work, the extraction procedures mimetized the conventional form of consumption of yerba mate, i.e, hot and cold water for green yerba mate (chimarrão and tererê, respectively) and hot water to toasted yerba mate (mate tea). For this reason, amounts of total phenolic compounds and flavonoids had been almost 3 times smaller than the amounts extracted by a mixture of methanol-water (Souza et al., 2015). Chimarrão presented the highest level of total phenolic compounds and flavonoids (111.46 ± 3.85 mg/g extract and 5.61±0.06 mg/g extract, respectively), followed by tererê (69.01 ± 4.72 and 1.00 ± 0.01 mg/g extract, respectively), and mate tea (64.35 ± 0.73 and 0.02±0.01 mg/g extract, respectively). The lowest amount of phenolic compounds in mate tea can be explained by the possible degradation of some compounds by the high temperatures necessary in the toasting process (Lima, Farah, King, de Paulis, & Martin, 2016).

After *in vitro* digestion total phenolic compounds of chimarrão, tererê and mate tea decreased by 74.69±5.48, 69.01±4.72 and 51.60±1.89 mg/g extract, respectively, representing

reductions of 33%, 24% and 20%, respectively. This behaviour is in agreement with findings in a previous study (Boaventura et al., 2015) and indicates that the transformation of the phenolic compounds may be influenced by pH changes and by interactions with other constituents during *in vitro* digestion. After colonic fermentation, no significant alterations in the total phenolic compounds were observed in chimarrão and tererê, while in mate tea, total phenolic compounds decreased by 34.64± 0.20 mg/g extract, what represents a reduction of 33%. The loss of phenolic compounds during the digestion process is unlikely due to interactions with digestive enzymes, but most probably caused by the chemical conditions prevailing during pancreatic digestion (Silberberg et al., 2006). The phenolic compounds are strongly sensible to the alkaline conditions found in the small intestine and the secretion of bile salts can cause alterations in the chemical structures resulting in new compounds, with different bioavailability and functional properties (Koehnlein et al., 2016).

The effects of *in vitro* digestion and colonic fermentation on the individual phenolic compounds of yerba mate beverages are shown in Figure 1. The most abundant phenolic compounds in the three beverages were salvianolic acid I (SA, a caffeic acid trimer), 5-*O*-caffeoiquinic acid (5CQA), 4-*O*-caffeoylquinic acid (4CQA), 3-*O*-caffeoylquinic acid (3CQA) and 3,5-*O*-dicaffeoyquinic acid (3,5 diCQA). Diminutions in the contents of all molecules was apparent. The decreases were more pronounced after *in vitro* digestion, than after *in vitro* colonic fermentation. Notably, on the other hand, a drastic reduction was observed for 5CQA in chimarrão and mate tea. According to Friedman & Jürgens (2000), some phenolic compounds are not stable at the alkaline pH found in the small intestine. For example, a previous study also described that the *in vitro* digestion of white and green tea caused a reduction in the content of phenolic compounds, mainly catechins, and the appearance of new compounds, probably flavonoid aglycones such as myricetin, quercetin and kaempferol and the appearance of ellagic acid, what suggests tannin degradation (Okello, Leylabi & McDougall, 2012).

Additionally, there is evidence that colon bacteria can convert phenolic compounds into several derivatives. For example, the CQAs can be converted into caffeic acid and dihydrocaffeic derivatives (Mills, Tzounis, Mottram, Gibson & Spencer, 2015). The colon bacteria can also be involved in other reactions such as sulfation and glucuronidation (Stalmach et al., 2010; Del Rio, Stalmach, Calani & Crozier, 2010).

3.2. Effects of in vitro digestion and colonic fermentation on the antioxidant activity of yerba mate beverages

Six antioxidant assays (DPPH, ABTS, FRAP, ORAC and TBARS assay and inhibition of the mitochondrial reactive oxygen species production) were carried out to evaluate the effects of *in vitro* digestion and colonic fermentation in yerba mate beverages (Figures 2 and 3). In general, the *in vitro* gastrointestinal and colonic fermentation caused a reduction, to a greater or lesser degree, in the antioxidant capabilities of the yerba mate beverages, except in the ABTS assay. Although the decreases in the antioxidant activities were statistically significant ($p \leq 0.05$) in several cases, the extracts maintained their antioxidant properties. The reduction of the antioxidant activities of green and toasted yerba mate after *in vitro* gastrointestinal digestion has been previously reported (Boaventura et al., 2015; Koehnlein et al., 2016).

The effects of *in vitro* digestion and *in vitro* colonic fermentation on the antioxidant activities depend essentially on two factors: the chemical nature of the antioxidants and the food matrix. Several works have described that the after *in vitro* digestion of cereals, legumes and vegetables extracts the total antioxidant capacities of extracts were significantly higher than those obtained with organic solvents or water (Liu, Glahn & Liu, 2004; Masisi, Beta & Moghadasian, 2016; Koehnlein et al., 2016). The higher values of the total antioxidant capacity after *in vitro* enzymatic digestion can be due, in part, to partial hydrolysis of the total phenolic compounds (Hsu, Hurang, Chen, Wenig & Tseng, 2004). In solid and complex food matrices, the antioxidant molecules, essentially phenolic compounds, can be conjugated to sugars, cell

wall polysaccharides, alcohols or amines (Masisi et al., 2016). As consequence, enzymatic hydrolysis of starch and proteins favours the release of antioxidant compounds (Gawlik-Dziki, Dziki, Baraniak & Lin, 2009). Contrarily, the gastrointestinal digestion can cause a reduction in the antioxidant activities of beverages, such as red wine, green tea, coffee and yerba mate. These results suggest that the phenolic compounds of food groups with solid and complex matrix are protected against the enzymatic action and alteration in pH during the digestion, what does not occur in liquid food matrices such as the beverages (Koehnlein et al., 2016). In these cases, the stability of the antioxidant molecules in the presence of digestive enzymes and changes of pH is crucial for antioxidant properties maintenance. In a recent study, only four (two types of plum, red bayberry and mango) from 33 tested fruits had their total antioxidant capacity improved after *in vitro* digestion (Chen, Chen, Zhao, Luo, Li, & Gao, 2014). An increase in the flavonoid contents of buckwheat and broccoli was observed after *in vitro* gastric digestion, suggesting stability of these compounds in the presence of pepsin. However, a reduction in the flavonoid contents was observed after pancreatic digestion (Gawlik-Dziki et al., 2009). A recent work evaluated the effect of *in vitro* digestion on the antioxidant activity of dietary supplements from pomegranate, milk thistle, green tea, grape seed, goji and acai, all of them as extracts. The authors concluded that, except for green tea and grape extracts no significant loss of antioxidant activity was observed during *in vitro* digestion (Henning et al., 2014).

3.3. Effects of in vitro digestion and colonic fermentation on the antibacterial activity of green and toasted yerba mate beverages

The green and toasted yerba mate extracts exhibited antibacterial activity against all Gram positive and Gram negative bacteria tested (Table 2). Also, all yerba mate extracts were more active against Gram positive bacteria, especially *Staphylococcus aureus*, *MRSA*-methicillin-resistant *Staphylococcus aureus*, and *MSSA*-methicillin-susceptible *Staphylococcus*

aureus. In general, the *in vitro* digestion and colonic fermentation barely affected the antimicrobial activities of the extracts. However, after *in vitro* digestion and colonic fermentation, the extracts were more active against *S. aureus*, MRSA and MSSA. Recent studies have shown that aqueous extracts of yerba mate present bactericidal and inhibitory effects on the growth of pathogenic bacteria, including MRSA (Burris, Davidson, Stewart & Harte, 2011; Burris, Higginbotham & Stewart, 2015). In general, the antibacterial activity is attributed to small phenolic molecules present in yerba mate (Heck & Mejia, 2007, Saleem et al., 2010). This attribution is confirmed by the antibacterial activity demonstrated for purified yerba mate phenolic compounds. For example, 3-*O*-caffeoylquinic acid (3CQA), one of the most abundant phenolic molecules in yerba mate, had strong antibacterial activity against *S. aureus* (MIC=40 µg/mL) and *E. coli* (MIC= 80 µg/mL) (Lou, Wang, Zhu, Ma & Wang, 2011). However, antibacterial activity has been reported for dialysed aqueous extracts of green yerba mate, what suggests that macromolecules such as proteins can be the responsible for this bioactivity (Burris et al., 2011). Yerba mate leaves possess around 26% of their dry weigh in proteins, and at least in part, these proteins may be extracted during aqueous extraction. Taking this into account, the antibacterial activities found in this work may also be due to the proteins and not only to the small molecules.

3.4. Effects of in vitro digestion and colonic fermentation in antiproliferative and cytotoxic actions of yerba mate beverages

The inhibition of proliferation of the four human cell lines (MCF-7, NCI-H460, HeLa and HepG2) and the cytotoxicity to non-tumor cells (PLP2) of yerba mate extracts submitted or not to *in vitro* digestion and colonic fermentation are presented in Table 3. All crude extracts showed cytotoxicity against HeLa cells. This cytotoxicity was slightly affected by *in vitro* digestion and colonic fermentation. No undigested or digested extracts presented cytotoxicity against HepG2 cells. Interestingly, the colonic fermentation improved the cytotoxicity of the

mate tea extract against all tumor cell lines, except HepG2. None of the tested extracts showed toxicity against normal (non-tumor) porcine liver primary cells (GI_{50}>400 µg/mL). Green yerba mate hydromethanolic extracts containing 28% of phenolic compounds were active against the same four tumor cell lines used in this work (Souza et al., 2015).

4. Conclusion

The results of this study demonstrate, for the first time, the effects of both *in vitro* digestion and *in vitro* colonic fermentation of yerba mate prepared in the three most common forms of consumption (chimarrão, tererê and mate tea). Despite the decrease in the phytochemicals content, yerba mate beverages maintained their functional properties such as antioxidant, antibacterial and antitumor activities after *in vitro* gastrointestinal digestion and *in vitro* colonic fermentation.

Conflict of interests

The authors declare no conflict of interests

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version

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Legend of Figures

Figure 1. Effects of *in vitro* gastrointestinal digestion and *in vitro* colonic digestion on the isolated phytochemicals of yerba mate beverages. A: chimarrão; B: tererê; C: mate tea. 3-*O*-caffeoylquinic acid 3-CQA); 4-*O*-caffeoylquinic acid (4-CQA); 5-*O*-caffeoylquinic acid (5-CQA); salvianolic acid I (SA); 1,3-*O*-dicaffeoylquinic acid (1,3diCQA); 3,5-*O*-dicaffeoylquinic acid (3,5diCQA); 3,4-*O*-dicaffeoylquinic acid (3,4diCQA); 4,5-*O*-dicaffeoylquinic acid (4,5diCQA); caffeic acid derivative (CAD); caffeic acid hexoside (CAH) and quercetin-3-*O*-rutinoside (Q3OR). Values with the same superscript symbol for each compound did not differ statistically from each other ($p < 0.05$).

Figure 2. Effects of *in vitro* gastrointestinal digestion and *in vitro* colonic digestion on the antioxidant activities of yerba mate beverages valuated by chemical methods. DPPH (A); ABTS (B); FRAP (C) and ORAC (D). Values with the same superscript symbol in the same group did not differ statistically from each other ($p < 0.05$).

Figure 3. Effects of *in vitro* gastrointestinal digestion and *in vitro* colonic digestion on the antioxidant activities of yerba mate beverages valuated by chemical-biological methods. TBARS assay (A) and inhibition of mitochondrial ROS generation (B). Values with the same superscript symbol in the same group did not differ statistically from each other ($p < 0.05$).

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and tentative identification of the phenolic compounds present in different preparations of *Ilex paraguariensis* A. St. Hil.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	4.9	325	353	191(100),179(46),173(3),161(1),135(7)	3-*O*-Caffeoylquinic acid[1]
2	5.2	275	341	191(8),179(100),173(5),161(5),135(5)	Caffeic acid derivative[2]
3	5.9	275	341	191(8),179(100),173(5),161(5),135(5)	Caffeic acid hexoside[2]
4	6.8	320	353	191(12),179(50),173(100),161(1),135(4)	4-*O*-Caffeoylquinic acid[1]
5	7.3	323	353	191(100),179(6),173(1),161(1),135(1)	5-*O*-Caffeoylquinic acid[1]
6	10.7	274	537	519(100),341(3),179(6),161(7),135()	Salvianolic acid I[3]
7	13.9	327	515	353(100),335(10),191(12),179(4),173(6),161(1),135(4)	1,3-*O*-Dicaffeoylquinic acid[1]
8	18.1	256/sh323	609	301 (100)	Quercetin-3-*O*-rutinoside[4]
9	19.4	325	515	353(100),335(10),191(12),179(4),173(6),161(1),135(4)	3,4-*O*-Dicaffeoylquinic acid[1]
10	20.9	325	515	353(100),335(1),191(1),179(1),173(1),161(1),135(5)	3,5-*O*-Dicaffeoylquinic acid[1]
11	21.5	266/sh332	593	285(100)	Kaempherol-3-*O*-rutinoside[5]
12	22.5	333	623	315(100)	Isorhametin-3-*O*-rutinoside[6]
13	23.5	327	515	353(100),335(5),191(1),179(2),173(3),161(1),135(5)	4,5-*O*-Dicaffeoylquinic acid[1]

Standard calibration curves: 1- chlorogenic acid (y = 208604x + 173056, R^2 = 0.9995); 2- caffeic acid (y = 388345x + 406369, R^2 = 0.9939); 3- rosmarinic acid (y = 191291x – 652903, R^2 = 0.999); 4- quercetin-3-*O*-rutinoside (y = 13343x + 76751, R^2 = 0.9998); 5- kampferol-3-*O*-rutinoside (y = 41843x + 220192, R^2 = 0.9998) and 6- isorhametin-3-*O*-glucoside (y = 11117x + 30861, R^2 = 0.9999).

Table 2. Antimicrobial activity (MIC values, mg/mL) of the crude extract (CE) after *in vitro* digestion (AIVDE) and after colonic fermentation extract (ACFE) of chimarrão, tererê and mate tea beverages (mean ± SD).

	chimarrão			**tererê**			**mate tea**		
	CE	AIVDE	ACLE	CE	AIVDE	ACFE	CE	AIVDE	ACFE
Gram negative bacteria									
Acinetobacter baumannii	2.500	0.625	2.500	2.500	1.250	2.500	1.250	1.250	1.250
Escherichia coli	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Escherichia coli	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Klebsiella pneumoniae	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Klebsiella pneumoniae	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Morganella morganii	2.500	1.250	2.500	2.500	1.250	1.250	2.500	2.500	2.500
Pseudomonas aeruginosa	5.000	5.000	10.000	5.000	2.500	10.000	2.500	2.500	5.000
Gram positive bacteria									
Enterococcus faecalis	5.000	5.000	5.000	5.000	5.000	10.000	5.000	5.000	5.000
Listeria monocytogenes	5.000	5.000	5.000	10.000	10.000	2.500	5.000	5.000	5.000
MRSA	0.625	0.312	0.312	1.250	0.312	0.625	2.500	2.500	0.312
MSSA	1.250	0.312	0.625	1.250	0.312	0.625	2.500	2.500	0.625
Staphylococcus aureus	1.250	1.250	0.625	1.250	0.625	1.250	0.625	1.250	0.625

MIC values correspond to the minimal sample concentration that inhibited the bacterial growth.

Table 3. Cytotoxicity of the crude extract (CE) after *in vitro* digestion (AIVDE) and after colonic fermentation extract (ACFE) of chimarrão, tererê and mate tea beverages (mean ± SD).

	chimarrão			**tererê**			**mate tea**			
	CE	AIVDE	ACFE	CEt	AIVDE	ACFE	CE	AIVDE	ACFE	Ellipticine
Human tumor cell lines (GI_{50} values, µg/mL)										
MCF-7 (breast carcinoma)	>400	>400	>400	>400	>400	>400	>400*	>400*	247±18**	1±0.1
NCI-H460 (non-small cell lung cancer)	>400	>400	>400	>400	>400	>400	>400*	>400*	284±24**	1±0.1
HeLa (cervical carcinoma)	238±5^{a}	143±12^{b}	232±10^{a}	249 ±15^{A}	217±20^{A}	219±4^{A}	162±11*	270±1**	224±11***	2±0.1
HepG2 (hepatocellular carcinoma)	>400	>400	>400	>400	>400	>400	>400	>400	>400	1±0.2
Non-tumor cells (GI_{50} values. µg/mL)										
PLP2	>400	>400	>400	>400	>400	>400	>400	>400	>400	3±0.7

Values with the same superscript symbol in the same line did not differ statistically from each other ($p < 0.05$).

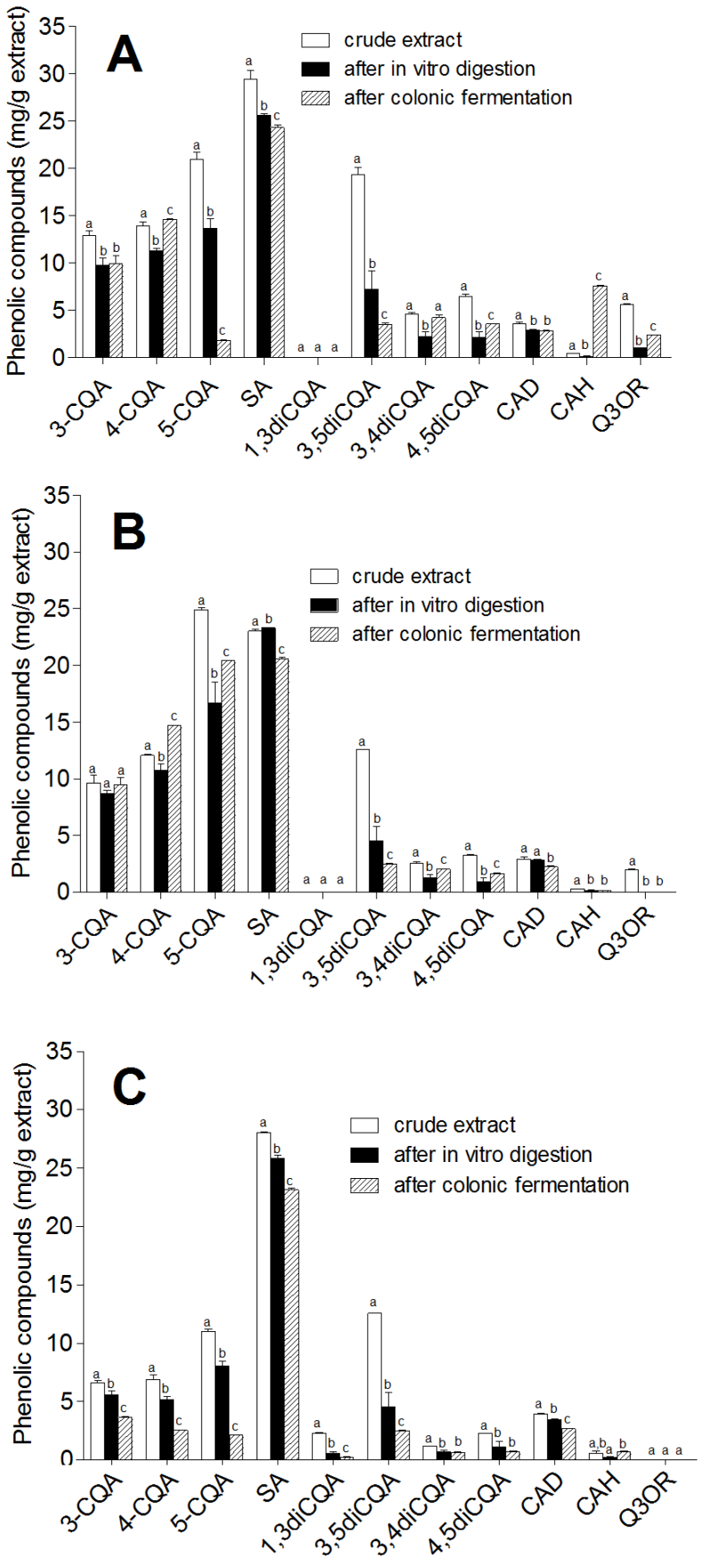
A
B
C
crude extract
after in vitro digestion
after colonic fermentation
Phenolic compounds (mg/g extract)
0
5
10
15
20
25
30
35
3-CQA
4-CQA
5-CQA
SA
1,3diCQA
3,5diCQA
3,4diCQA
4,5diCQA
CAD
CAH
Q3OR

Figure 1

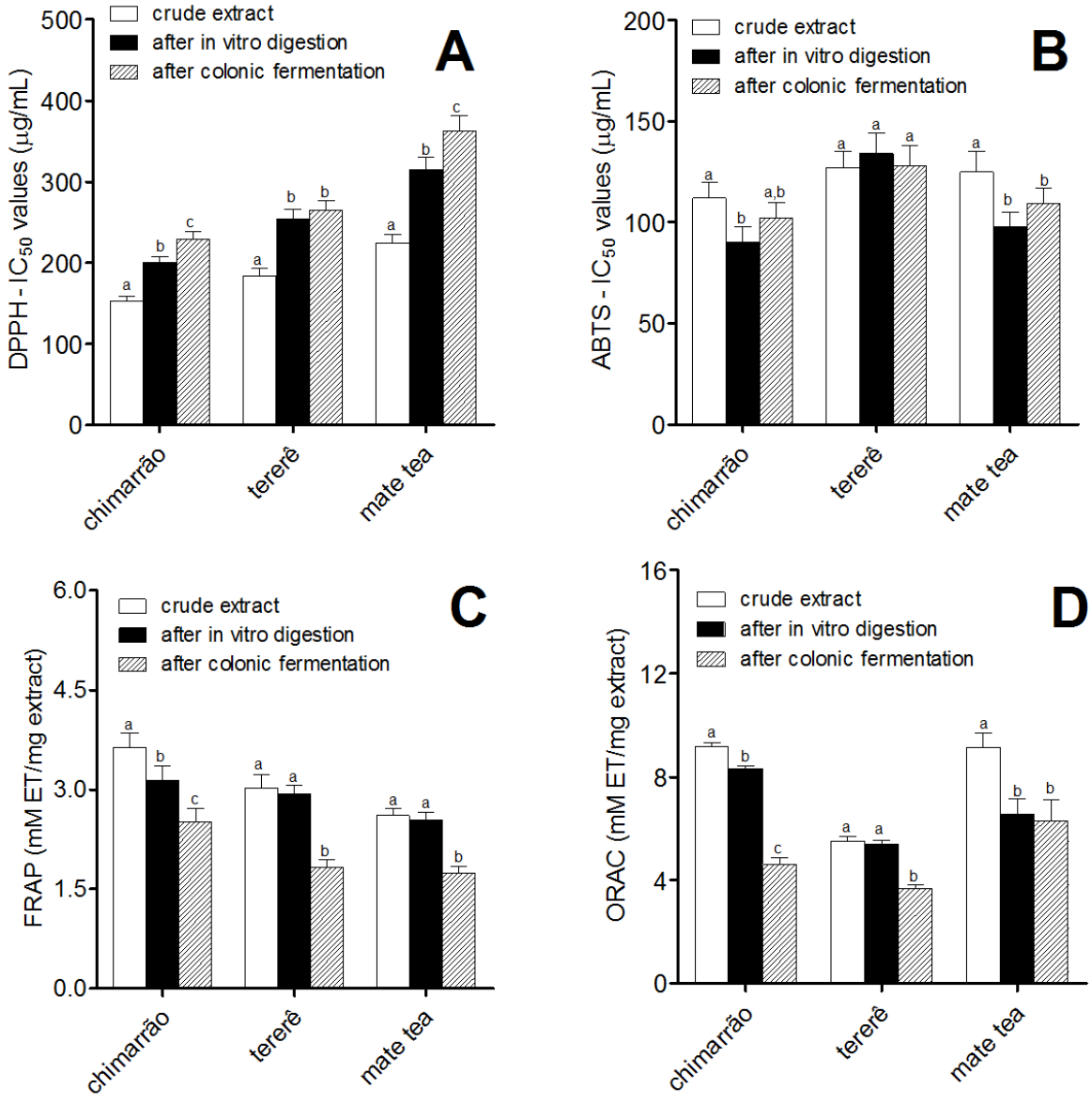
A
crude extract
after in vitro digestion
after colonic fermentation
DPPH - IC50 values (µg/mL)
500
400
300
200
100
0
chimarrão
terere
mate tea
B
ABTS - IC50 values (µg/mL)
200
150
100
50
0
C
FRAP (mM ET/mg extract)
6.0
4.5
3.0
1.5
0.0
D
ORAC (mM ET/mg extract)
16
12
8
4
0

Figure 2

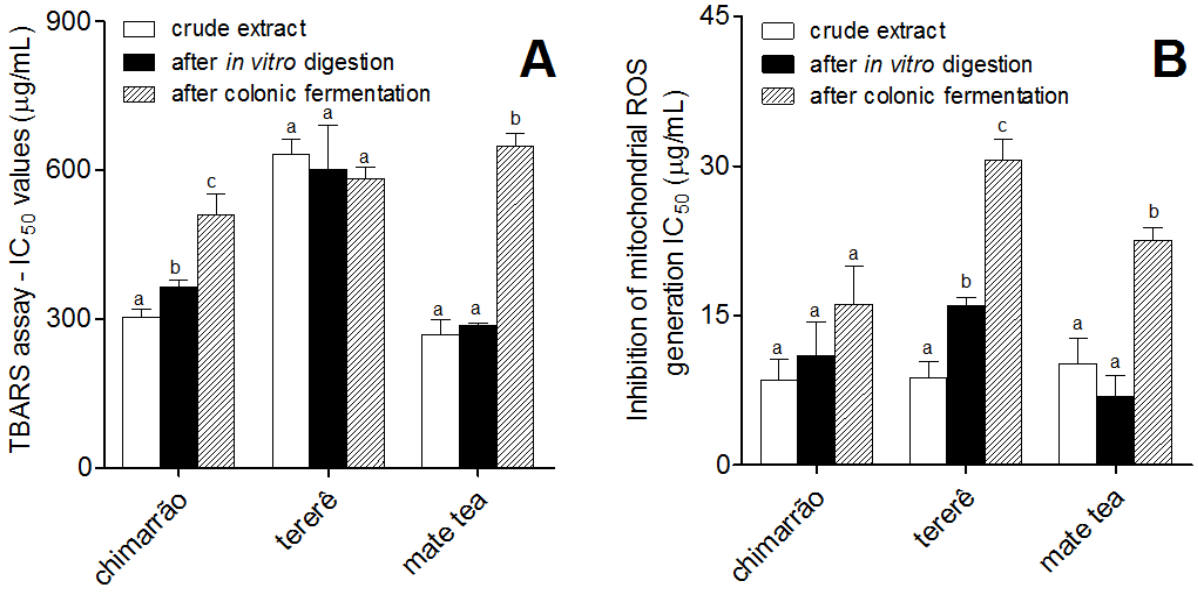
A
crude extract
after *in vitro* digestion
after colonic fermentation
TBARS assay - IC_{50} values (µg/mL)
900
600
300
0
a
b
c
a
a
a
a
a
b
chimarrão
tererê
mate tea
B
crude extract
after *in vitro* digestion
after colonic fermentation
Inhibition of mitochondrial ROS generation IC_{50} (µg/mL)
45
30
15
0
a
a
a
a
b
c
a
a
b
chimarrão
tererê
mate tea

Figure 3

SUPPLEMENTARY MATERIAL

Effects of *in vitro* digestion and *in vitro* colonic fermentation on stability and functional properties of yerba mate (*Ilex paraguariensis* A. St. Hil.) beverages

Vanesa G. Correa, Geferson A. Gonçalves, Anacharis B. de Sá-Nakanishi, Isabel C. F. R. Ferreira, Lillian Barros, Maria I. Dias, Eloá A. Koehnlein, Adelar Bracht, Rosane M. Peralta.

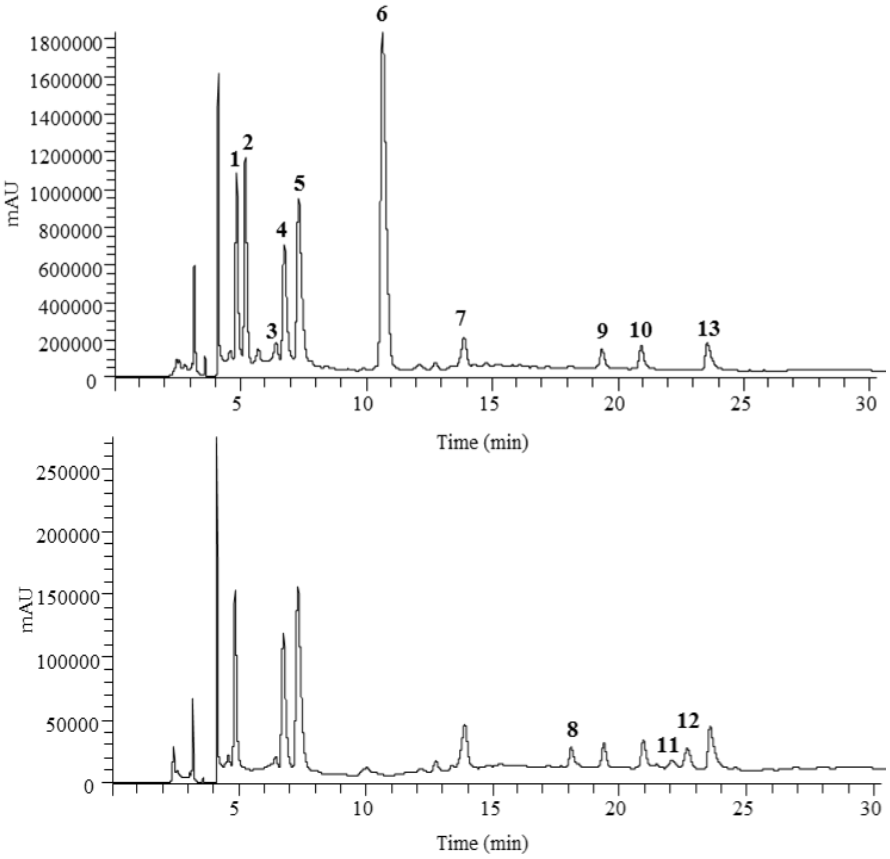

Figure S1. HPLC phenolic profile of mate tea crude extract obtained at 280 nm (A) and 370 nm (B) for phenolic acids and flavonols, respectively.