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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 G. Correa [a,b], Geferson A. Gonçalves [a,b], Anacharis B. de Sá-Nakanishi [a,b], Isabel C.F.R. Ferreira [c,*], Lillian Barros [c,d], Maria I. Dias [c,d], Eloá A. Koehnlein [e], Cristina G.M. de Souza [b], Adelar Bracht [a,b], Rosane M. Peralta [a,b,*]

[a] *Department of Biochemistry, State University of Maringá, Paraná, Brazil*
[b] *Graduate Program in Food Science, State University of Maringá, Paraná, Brazil*
[c] *Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança (IPB), Campus de Santa Apolonia, Bragança, Portugal*
[d] *Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials (LSRE-LCM), Polytechnic Institute of Bragança, Campus de Santa Apolónia, Bragança, Portugal*
[e] *Department of Nutrition, Federal University of South Border, Realeza, Paraná, Brazil*

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ABSTRACT

Yerba mate (*Ilex paraguariensis*) is a plant that grows naturally in South America. From its leaves and thin stems different kinds of beverages are prepared (chimarrão, tererê and tea mate), all of them rich in bioactive substances. 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. The phenolic chromatographic profile 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 maintain their functional properties such as antioxidant, antibacterial and antitumoral activities.

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1. Introduction

The yerba mate (YM) (*Ilex paraguariensis* A. St. Hil.) is a plant that grows naturally in Paraguay, Uruguay, Argentina and Brazil. The powder of YM leaves and thin stems is used for preparation of several stimulant drinks. The three most important forms of consumption are *chimarrão* (hot water extract of green dried leaves; *mate* in Spanish speaking countries), *tererê* (cold water extract of green dried leaves) and *mate tea* (hot water extract of toasted leaves) (Bracesco, Sanchez, Contreras, Menini, & Gugliucci, 2011; Lima et al., 2014a).

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 energy 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 the lipid profile and blood circulation (Kim, Oh, Kim, Chae, & Chae, 2015; Lima et al., 2014b). It is also used as diuretic and anti-rheumatic (Isolabella et al., 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).

* Corresponding authors at: Department of Biochemistry, State University of Maringá, Paraná, Brazil (R.M. Peralta) and Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança (IPB), Campus de Santa Apolonia, Bragança, Portugal (I.C.F.R. Ferreira).

E-mail addresses: iferreira@ipb.pt (I.C.F.R. Ferreira), rmperalta@uem.br, rosanemperalta@gmail.com (R.M. Peralta).

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Yerba mate is known to be rich in phenolic acids such as caffeic acid (CA) and chlorogenic acid (CGA) and their derivatives in addition to 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 as consequence of pH alterations, enzymatic actions, and the metabolic activity of the intestinal microbiota (Boaventura et al., 2015; Correa-Betanzo et al., 2014). Time and temperature of digestion can 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 reached 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), fetal 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. Preparation of beverages and lyophilisates

Raw and toasted yerba mate were obtained from reliable commercial sources and producers in Southern 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 °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 °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.

2.3. In vitro digestion

In vitro gastrointestinal digestion was simulated as previously described (Koehnlein et al., 2016). Briefly, 13 g of each lyophilisate 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. Then, the pH was adjusted to 1.2 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 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.

2.4. 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 collection the material was homogenized with the culture medium at a ratio of 1:10 (w/v). The bottles were bubbled again with nitrogen for the same time as for the previous one and sealed airtight. Afterwards, they were incubated at 37 °C 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.

2.5. Analysis of phenolic compounds

The lyophilisates of each beverage were re-dissolved in water and analyzed by high-performance liquid chromatography with photodiode array detection–electrospray ionization multiple-stage mass spectrometry (HPLC-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.

2.6. Evaluation of antioxidant activity

Six different methods were used to evaluate the antioxidant activity: reduction power of the ferric ion (FRAP), oxygen radical absorbance radical (ORAC), reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), reduction of the 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonate) cation (ABTS), inhibition of the production of thiobarbituric acid reactive substances (TBARS) assay and inhibition of the mitochondrial reactive oxygen species (ROS) production. Successive dilutions of the stock solution were made and used for assaying the antioxidant activity of the samples. The lyophilisate concentrations (mg/mL) providing 50% of antioxidant activity were calculated from the graphs of antioxidant activity against the sample concentrations. Trolox was used as a positive control.

FRAP and ORAC were evaluated as previously described (Koehnlein et al., 2016). Standard curves were constructed with trolox ($r^2 = 0.99$) and the results were expressed as mmol trolox equivalents (TE)/ mg lyophilisate material.

The DPPH and the ABTS assays were conducted as described previously (Correa et al., 2015). The percentage of DPPH and ABTS discoloration was calculated using the following equation: $[(A_{CONTROL} - A_{SAMPLE})/A_{CONTROL}] \times 100$. The results were expressed as IC_{50} values (sample concentration providing 50% of antioxidant activity).

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

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

2.7. Antibacterial activity evaluation

The lyophilisates 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. Minimum inhibitory concentration (MIC) determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay (Kuete et al., 2011a, 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 lyophilisates, 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).

2.8. Evaluation of cytotoxic properties

The lyophilisates 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).

2.9. 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 the 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 shown in Table 1. Thirteen phenolic compounds were identified, ten phenolic acids (chlorogenic, caffeic and rosmarinic acids derivatives), and three flavonoids, namely quercetin-3-*O*-rutinoside, kaempherol-3-*O*-rutinoside and isorhametin-3-*O*-rutinoside. From the 13 molecules, eleven (compounds 1–6, 8–11 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 this 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 but only in trace amounts in green yerba mate (chimarrão and tererê).

Alcoholic and hydro-alcoholic extracts of *I. paraguariensis* leaves have been described as being rich in chlorogenic acid, a group of compounds comprising hydroxycinnamates, such as caffeic, ferulic and *p*-coumaric acids. They are 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

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)	λ_{max} (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(2)	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).

procedures mimetized the conventional form of consumption of yerba mate, i.e., hot water and water at room temperature for green yerba mate (chimarrão and tererê, respectively) and hot water for toasted yerba mate (mate tea). For this reason, the amounts of total phenolic compounds and flavonoids were 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 lyophilisate and 5.61 ± 0.06 mg/g lyophilisate, respectively), followed by tererê (69.01 ± 4.72 and 1.00 ± 0.01 mg/g lyophilisate, respectively), and mate tea (64.35 ± 0.73 and 0.02 ± 0.01 mg/g lyophilisate, 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 the total phenolic compounds of chimarrão, tererê and mate tea decreased to 74.69 ± 5.48, 69.01 ± 4.72 and 51.60 ± 1.89 mg/g lyophilisate, respectively. These final contents represent reductions of 33%, 24% and 20%, respectively. This behaviour is in agreement with the findings of 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 lyophilisate, 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 highly 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 bioavailabilities 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 Table 2. 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 were

Table 2
Phenolic compounds quantification (mg/g) in the beverages, after digestion and after colonic fermentation (mean ± SD).

Peak	Chimarrão			Tererê			Chá mate		
	Crude extract	*In vitro* digestion	Colonic fermentation	Crude extract	*In vitro* digestion	Colonic fermentation	Crude extract	*In vitro* digestion	Colonic fermentation
1	12.88 ± 0.49^{A}	9.74 ± 0.75^{B}	9.89 ± 0.89^{B}	9.61 ± 0.67^{a}	8.64 ± 0.34^{a}	9.48 ± 0.65^{a}	6.64 ± 0.17*	5.58 ± 0.32**	3.65 ± 0.08***
2	3.57 ± 0.16^{A}	2.87 ± 0.06^{B}	2.81 ± 0.05^{B}	2.88 ± 0.21^{a}	2.79 ± 0.09^{a}	2.23 ± 0.07^{b}	3.92 ± 0.10*	3.48 ± 0.02**	2.67 ± 0.03***
3	0.38 ± 0.01^{A}	0.10 ± 0.04^{B}	7.56 ± 0.07^{C}	0.27 ± 0.01^{a}	0.10 ± 0.07^{b}	0.12 ± 0.01^{b}	0.56 ± 0.21*	0.23 ± 0.09**	0.71 ± 0.06*
4	13.88 ± 0.44^{A}	11.23 ± 0.34^{B}	14.58 ± 0.06^{C}	12.05 ± 0.09^{a}	10.74 ± 0.59^{b}	14.68 ± 0.06^{c}	6.88 ± 0.39*	5.16 ± 0.29**	2.52 ± 0.01***
5	20.93 ± 0.73^{A}	13.64 ± 1.05^{B}	1.79 ± 0.04^{C}	24.89 ± 0.17^{a}	16.69 ± 1.80^{b}	20.40 ± 0.05^{c}	10.99 ± 0.23*	8.01 ± 0.45**	2.16 ± 0.02***
6	29.44 ± 0.89^{A}	25.64 ± 0.20^{B}	24.29 ± 0.30^{C}	23.01 ± 0.20^{a}	23.31 ± 0.05^{b}	20.58 ± 0.14^{c}	28.07 ± 0.03*	25.80 ± 0.32**	23.15 ± 0.16***
7	nd	nd	nd	nd	nd	nd	2.3 ± 0.05*	0.54 ± 0.20**	0.19 ± 0.09***
8	5.61 ± 0.06^{A}	1.00 ± 0.01^{B}	2.36 ± 0.06^{C}	1.96 ± 0.04^{a}	tr^{b}	tr^{b}	tr	tr	nd
9	4.59 ± 0.18^{A}	2.23 ± 0.49^{B}	4.22 ± 0.27^{A}	2.55 ± 0.10^{a}	1.29 ± 0.22^{b}	2.02 ± 0.03^{c}	1.18 ± 0.02*	0.69 ± 0.15**	0.65 ± 0.05**
10	19.34 ± 0.72^{A}	7.17 ± 2.01^{B}	3.46 ± 0.19^{C}	12.55 ± 0.05^{a}	4.51 ± 1.30^{b}	2.49 ± 0.05^{c}	1.52 ± 0.05*	0.96 ± 0.25**	0.22 ± 0.02***
11	tr	tr	tr	tr	tr	tr	tr	tr	nd
12	tr	tr	tr	tr	nd	tr	0.02 ± 0.01*	tr**	nd**
13	6.45 ± 0.24^{A}	2.07 ± 0.65^{B}	3.53 ± 0.06^{C}	3.27 ± 0.03^{a}	0.93 ± 0.36^{b}	1.62 ± 0.04^{c}	2.28 ± 0.03*	1.14 ± 0.47**	0.72 ± 0.04**
Total phenolic acids	111.46 ± 3.85^{A}	74.69 ± 5.48^{B}	72.13 ± 1.81^{B}	91.08 ± 0.04^{a}	69.01 ± 4.72^{b}	73.62 ± 0.34^{b}	64.35 ± 0.73*	51.60 ± 1.89**	34.64 ± 0.20***
Total flavonoids	5.61 ± 0.06^{A}	1.00 ± 0.01^{B}	2.36 ± 0.06^{C}	1.96 ± 0.04^{a}	tr^{b}	tr^{b}	0.02 ± 0.01*	tr**	tr**

Values with different superscript symbol in the same line for the same group differ statistically from each other ($p < 0.05$).

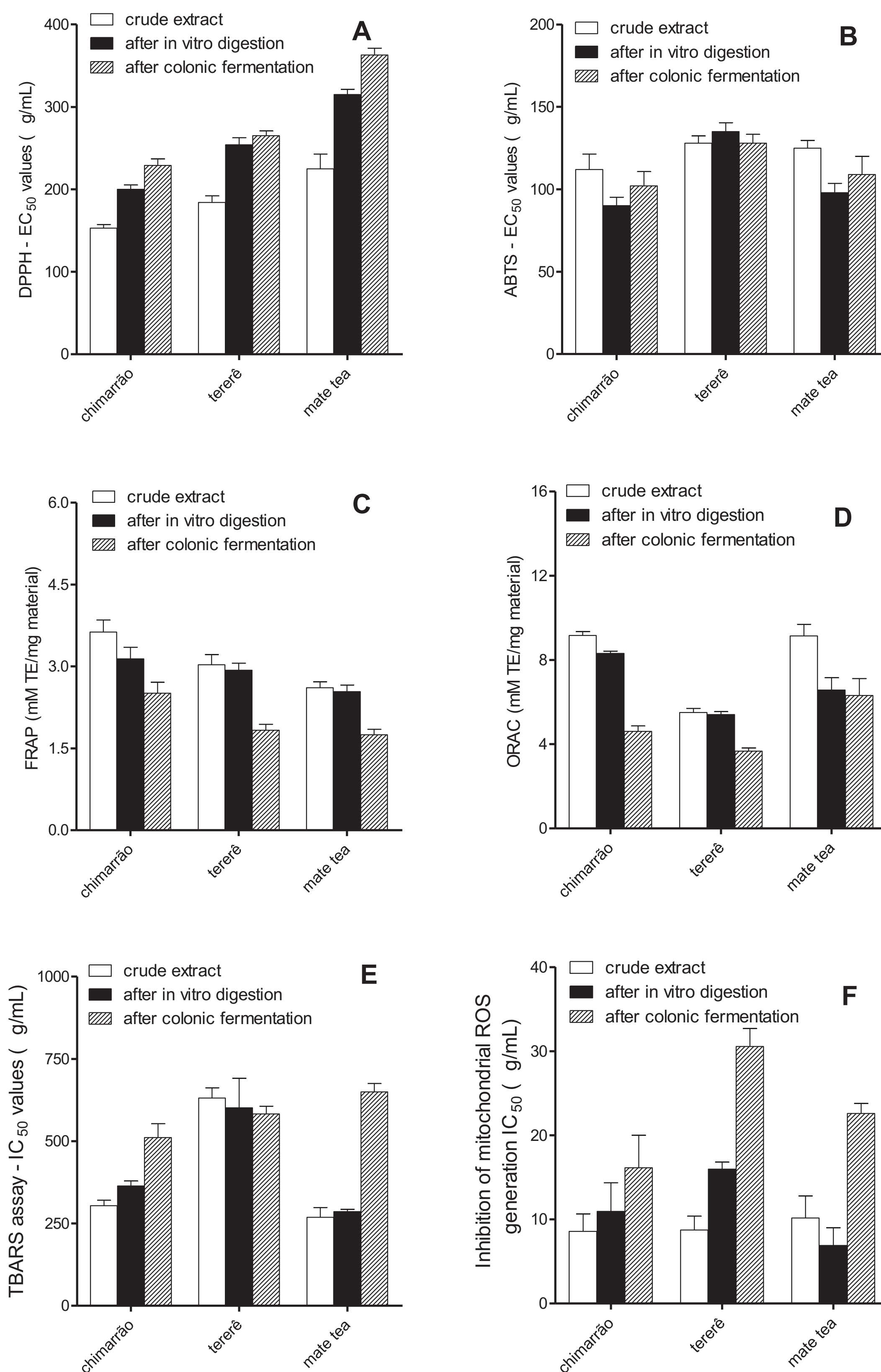

Fig. 1. Effects of *in vitro* gastrointestinal digestion and *in vitro* colonic digestion on the antioxidant activities of yerba mate beverages. Chemical methods: DPPH (A); ABTS (B); FRAP (C) and ORAC (D). Chemico-biological methods: TBARS assay (E) and inhibition of mitochondrial ROS generation (F).

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 and Jürgens (2000), some phenolic compounds are not stable at the alkaline pH prevailing 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. The latter are probably flavonoid aglycones such as myricetin, quercetin and kaempferol and also ellagic acid, what suggests tannin degradation (Okello, Leylabib, & 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 (Del Rio, Stalmach, Calani, & Crozier, 2010; Stalmach et al., 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 assays and inhibition of the mitochondrial reactive oxygen species production) were carried out to evaluate the effects of *in vitro* digestion and colonic fermentation on the yerba mate beverages (Fig. 1). Different methods have been used due to the fact that antioxidant compounds can act by distinct mechanisms, each having its specific target within the reaction matrix. Different chemical reactivities may, thus, result in distinct degrees of antioxidant capacity in the various chemical tests (Fernández-Moriano, González-Burgos, Divakar, Crespo, & Gómez-Serranillos, 2016).

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 studies have described that the total antioxidant capacities of extracts obtained from cereals and legumes were significantly higher after *in vitro* digestion, an observation valid for extracts obtained with both water and organic solvents (Koehnlein et al., 2016; Liu, Glahn, & Liu, 2004; Masisi, Beta, & Moghadasian, 2016). The higher 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). In 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 the maintenance of their antioxidant capacities. In a recent study, only four (two types of plum, red bayberry and mango) out of 33 tested fruits had their total antioxidant capacities improved after *in vitro* digestion (Chen et al., 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 found that, except for green tea and grape extracts, no significant losses of antioxidant activities occurred 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 beverages exhibited antibacterial activity against all tested Gram positive and Gram negative bacteria (Table 3). The antibacterial activities, of all yerba mate

Table 3
Antimicrobial activities (MIC values, mg/mL) of chimarrão, tererê and mate tea beverages before (CE) and after *in vitro* digestion (AIVDE) and after colonic fermentation (ACFE) (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. MRSA: methicillin-resistant *Staphylococcus aureus*. MSSA: methicillin-sensitive *Staphylococcus aureus*.

Table 4
Cytotoxicity of chimarrão, tererê and mate tea beverages before (CE) and after *in vitro* digestion (AIVDE) and after colonic fermentation (ACFE) (mean ± SD).

	Chimarrão			Tererê			Mate tea			Ellipticine
	CE	AIVDE	ACFE	CE	AIVDE	ACFE	CE	AIVDE	ACFE	
Human tumor cell lines (GI_{50} values, μg/mL)										
MCF-7 (breast carcinoma)	>400	>400	>400	>400	>400	>400	$>400^{*}$	$>400^{*}$	$247 \pm 18^{**}$	1 ± 0.1
NCI-H460 (non-small cell lung cancer)	>400	>400	>400	>400	>400	>400	$>400^{*}$	$>400^{*}$	$284 \pm 24^{**}$	1 ± 0.1
HeLa (cervical carcinoma)	238 ± 5^{a}	143 ± 12^{b}	232 ± 10^{a}	249 ± 15^{A}	217 ± 20^{A}	219 ± 4^{A}	$162 \pm 11^{*}$	$270 \pm 1^{**}$	$224 \pm 11^{***}$	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 different superscript symbol in the same line for the same group differ statistically from each other ($p < 0.05$). Cytotoxicity results are expressed in GI_{50} values corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. PLP2: porcine liver primary cells.

beverages, however, were more pronounced 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 beverages. However, after *in vitro* digestion and colonic fermentation, the beverages were more active against *S. aureus*, MRSA and MSSA. Recent studies have shown that aqueous extracts of yerba mate present bactericidal effects and inhibit 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 & de 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, which suggests that macromolecules such as proteins and polysaccharides can be responsible for this bioactivity (Burris et al., 2011). Yerba mate leaves possess around 26% of their dry weigh in proteins, and these proteins may, at least in part, be extracted during beverage preparation. Taking this into account, the antibacterial activities found in this work may also be due to the proteins and not only the small molecules.

3.4. Effects of in vitro digestion and colonic fermentation on 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 the yerba mate beverages submitted or not to *in vitro* digestion and colonic fermentation are presented in Table 4. Prior to *in vitro* digestion and colonic fermentation all beverages showed cytotoxicity against HeLa cells. This cytotoxicity was slightly affected by the *in vitro* digestion and colonic fermentation. No undigested or digested beverages presented cytotoxicity against HepG2 cells. Interestingly, the colonic fermentation improved the cytotoxicity of the mate tea beverage against all tumor cell lines, except HepG2. None of the tested beverages 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

This study analyzed, for the first time, the effects of both *in vitro* digestion and *in vitro* colonic fermentation of yerba mate prepared according to the three most common forms of consumption (chimarrão, tererê and mate tea). Despite the decrease in the content phytochemicals, the yerba mate beverages maintain 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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