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Trace elements in ready-to-drink ice tea: Total content, *in vitro* bioaccessibility and risk assessment

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ABSTRACT

Tea is one of the most consumed non-alcoholic beverages in world and it has been frequently associated to health benefits. Besides its nutrient composition, non-essential trace elements associated with toxic effects may also be present. Ever since food components undergo biotransformation process along gastrointestinal tract after ingestion, it is important to evaluate both total and bioavailable content of trace elements. Therefore, this study aimed to provide comprehensive data concerning the influence of the *in vitro* digestion on sixteen trace elements present in ready-to-drink ice tea (black, green, mate and white tea). Essential minerals (Co, Cr, Cu, Fe, Mn, Se and Zn) and inorganic contaminants (Al, As, Cd, Li, Ni, Pb, Sb, Sn and Sr) contents were determined by ICP OES after microwave acid digestion. Bioaccessibility evaluation was carried out by simulating the gastric (pepsin) and intestinal juice (pancreatin and bile salts) and bioavailability used Caco-2 cells culture as an intestinal epithelial model. Moreover, tannins were evaluated by UV–VIS spectroscopy. Multivariate analysis allowed classifying ice tea samples in three groups, based on their trace elements profile. Al, Cu, Sr, Mn and Zn bioaccessible fractions corresponded to, approximately, 40–60% of their total content. For Mn, bioaccessibility and bioavailability presented the same pattern (green ice tea > black ice tea > mate ice tea) whilst Sr bioavailability in green tea were 50% higher than in black tea samples.

1. Introduction

One of the most consumed non-alcoholic beverages in world, tea is an ancient beverage traditionally prepared by infusion of *Camellia sinensis* leaves (Preedy, 2013). Ready-to-drink ice tea represents an increasing market in Brazil. In 2017, the production was superior to 116 million liters and this category also includes beverages made by tea and herbal tea (especially mate tea, *Ilex paraguariensis*) with fruit flavors such as lemon, peach and lychee juice (ABIR, 2020).

Tea is usually associated with health benefits due to its composition. It has been reported as source of several nutrients, such as vitamins, minerals and antioxidant compounds, such as polyphenols (Milani, Morgano, & Cadore, 2016; Schmite et al., 2019; Pohl, Szymczycha-Madeja, & Welna, 2020). The presence of trace elements in tea is usually related to environmental pollution, soil, irrigation water and stainless steel equipment used in food industries. Even though trace elements, such as Co, Cr, Cu, Fe, Mn, Se and Zn are indispensable for human nutrition, some trace elements with non-essential role may be found in tea beverages. These compounds are known as inorganic contaminants, such as Al, As, Cd, Li, Ni, Pb, Sb, Sn and Sr and may cause toxic effects even at low levels, which include cancer, gastrointestinal and neurological disorders (Zhang et al., 2018; Milani, Silvestre, Morgano, & Cadore, 2019).

Ever since food components undergo biotransformation process after ingestion, it is important considering the bioaccessible and bioavailable fractions to perform a more accurate risk assessment relating to inorganic contaminants. These fractions are usually defined as the maximum fraction of a compound released from food after digestion and available for absorption (bioaccessible) and the fraction of this compound that can be absorbed by the intestine for biological functions (uptake or absorbable fraction) (Moreda-Piñeiro et al., 2011; Sanches, Peixoto, & Cadore, 2020). Both of these fractions can be studied by simulation of *in vitro* gastrointestinal tract combined with an intestinal epithelial model, such as Caco-2 cells culture which is recognized as a preliminary

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screening for bioavailability (Do Nascimento da Silva & Cadore, 2019). Some antinutritional factors may interfere in trace elements and mineral absorption (Siqueira Silva, Rebellato, Caramês, Greiner, & Pallone, 2020). In vegetables, one of the main factors is the tannins, also known as flavanols or flavan-3-ols. This group of phenolic compounds is characterized by its water soluble behavior and the ability to form insoluble complex with proteins and trace elements, such as iron-polyphenol chelates (Granato, Santos, Maciel, & Nunes, 2016; Casanova & Arturo, 2012; Erdemir, 2018). Although phenolic compounds are usually associated to several health benefits, it is important to consider their negative influence on trace elements absorption (Do Nascimento da Silva et al., 2015).

In vitro digestion models applied for minerals evaluation in tea samples are usually consisted in two steps, simulating the gastric and the intestinal phases. Erdemir (2018) performed an evaluation of the bioaccessibility of Mg, Mn and Fe in tea samples from Turkey using an *in vitro* digestion model with the enzymes pepsin and pancreatin. Authors reported bioaccessible levels in black, earl grey and green tea infusions ranging from 23% to 105%, 9% to 100% and 66% to 84% for Fe, Mg and Mn, respectively. Schmite et al. (2019) reported a study of Al, Cd, Cu and Pb levels in yerba mate from Brazil employing an *in vitro* digestion model with the enzymes pepsin, pancreatin and bile salts. The bioaccessible fractions reported by those authors in mate cold and hot infusions ranged from ND to 95% for all trace elements. Szymczycha-Madeja, Welna, and Pohl (2020) reported a study combining *in vitro* digestion and dialysis assay (dialysis membrane tubing) for trace elements bioaccessibility assessment for black and green infusions. The authors reported dialyzed fractions ranging from 1.2% to 46.3% for Al, Ba, Ca, Cu, Fe, Mg, Mn, Ni, Sr and Zn.

Though only few studies were reported combining the *in vitro* digestion with Caco-2 cells model for minerals evaluation in beverages samples. Comprehensive studies about bioaccessibility and absorption for minerals were reported in literature for Ca, Fe, Zn in Ca-fortified milk (Perales, Barberá, Lagarda, & Farré, 2006); for Fe in milk and soy-based yogurts (Laparra, Tako, Glahn, & Miller, 2008); for Ca and P in milk-based fruit beverages (Cilla et al., 2011) and Cu, Fe, Mn, Mo, Se and Zn in selenium-enriched lettuce (Do Nascimento da Silva & Cadore, 2019).

Despite the importance of studies regarding the effect of *in vitro* digestion in elements from beverages, to the best of our knowledge, no comprehensive studies were reported in literature concerning bioaccessibility and bioavailability of trace elements present in ready-to-drink ice tea. Thus, the present work aims: i) to evaluate the content of sixteen trace elements (Al, As, Cd, Co, Cr, Cu, Fe, Li, Mn, Ni, Pb, Sb, Se, Sn, Sr and Zn) in ready-to-drink ice tea; ii) to study the effect of *in vitro* digestion in the bioavailability of these trace-elements in ice teas and the influence of tannins and iii) to estimate the mineral dietary intake and potential risk related to inorganic contaminants exposure from ice tea consumption.

2. Materials and methods

2.1. Samples

Ready-to-drink ice teas were acquired in markets from Campinas (Brazil): green ice tea (n = 6), white ice tea (n = 3), mate ice tea (n = 6) and black ice tea (n = 4), totalizing 19 samples. Sampling considered the available types, flavors and, at least two different batches from the four main brands: *green tea* (T1, T18), with lemon and peppermint (T4, T14) and red fruits flavor (T7, T15); *white tea* (T2) and with lychee flavor (T3, T19); *mate tea* (T8, T17), with lemon (T5, T16) and peach flavor (T6, T13); *black tea* with lemon (T9, T12) and peach flavor (T10, T11). From labelling (Table 1), main ingredients were water, tea (powder or extract), fruit juice, flavor, acidulants (such as ascorbic acid, citric acid, malic acid, sodium citrate, and potassium citrate), sugar and/or sugar substitutes (such as sodium cyclamate). Carbohydrates contents were, approximately, 18 g / 200 mL for ice teas with sugar in composition (mate and black teas) and not significant for ice teas with sugar substitutes (green and white teas). Samples were maintained in their original flasks, and when opened, they were immediately analyzed.

Table 1
Main ingredients described in ready-to-drink ice teas labelling.

Ice tea sample		Main ingredients
T1	Green tea	Water, powdered green tea (*Camellia sinensis*), vitamin C, sodium citrate, potassium citrate, citric acid, malic acid, lemon flavor and sugar substitutes.
T18	Green tea	Water, powdered green tea (*Camellia sinensis*), vitamin C, sodium citrate, potassium citrate, citric acid, malic acid, lemon flavor and sugar substitutes.
T4	Green tea with lemon and peppermint	Water, green tea extract (*Camellia sinensis*), citric acid, sodium citrate, ascorbic acid, fibers, lemon flavor and sugar substitutes.
T14	Green tea with lemon and peppermint	Water, green tea extract (*Camellia sinensis*), citric acid, sodium citrate, ascorbic acid, fibers, lemon flavor and sugar substitutes.
T7	Green tea with red fruits	Water, green tea extract (*Camellia sinensis*), citric acid, sodium citrate, ascorbic acid, fibers, red fruits flavor and sugar substitutes.
T15	Green tea with red fruits	Water, green tea extract (*Camellia sinensis*), citric acid, sodium citrate, ascorbic acid, fibers, red fruits flavor and sugar substitutes.
T2	White tea	Water, powdered white tea (*Camellia sinensis*), vitamin C, citric acid, malic acid, potassium citrate, sodium citrate and sugar substitutes.
T3	White tea with lychee	Water, powdered white tea (*Camellia sinensis*), vitamin C, citric acid, malic acid, potassium citrate, sodium citrate, lychee flavor and sugar substitutes.
T19	White tea with lychee	Water, powdered white tea (*Camellia sinensis*), vitamin C, citric acid, malic acid, potassium citrate, sodium citrate, lychee flavor and sugar substitutes.
T8	Mate tea	Water, sugar and mate extract (*Ilex paraguariensis*).
T17	Mate tea	Water, sugar and mate extract (*Ilex paraguariensis*), citric acid and ascorbic acid.
T5	Mate tea with lemon	Water, sugar, mate extract (*Ilex paraguariensis*) and lemon juice.
T16	Mate tea with lemon	Water, sugar, mate extract (*Ilex paraguariensis*) and lemon juice.
T6	Mate tea with peach	Water, sugar, mate extract (*Ilex paraguariensis*), peach juice, ascorbic acid, citric acid and flavors.
T13	Mate tea with peach	Water, sugar, mate extract (*Ilex paraguariensis*), peach juice, ascorbic acid, citric acid and flavors.
T9	Black tea with lemon	Water, sugar, lemon juice, black tea extract (*Camellia sinensis*), citric acid and phosphoric acid.
T12	Black tea with lemon	Water, sugar, lemon juice, black tea extract (*Camellia sinensis*), citric acid and phosphoric acid.
T10	Black tea with peach	Water, sugar, peach juice, black tea extract (*Camellia sinensis*), citric acid and phosphoric acid.
T11	Black tea with peach	Water, sugar, peach juice, black tea extract (*Camellia sinensis*), citric acid and phosphoric acid.

2.2. Reagents

Water and nitric acid were purified using a sub-boiling distiller (Berghof, Eningen, Germany) and a reverse osmosis system (Gehaka, São Paulo, Brazil), respectively. Analytical curves were obtained by dilutions of 1000 mg L^{-1} multielementar standard solution (Merck, Darmstadt, Germany) and 1000 mg L^{-1} Se and Sb standard solutions (Fluka, Steinheim, Germany) in the following ranges: 1.0–1000 μg L^{-1} for Cu, Sn and Zn; 2.5–5000 μg L^{-1} for Al, Fe and Mn; 1.0–500 μg L^{-1} for As, Cd, Co, Cr, Li, Ni, Pb, Sb, Se, and Sr.

For bioaccessibility study, bile extract (from porcine, cod. B8631), pepsin (from porcine gastric mucosa, 480 U mg^{-1}, cod. P7000) and pancreatin (from porcine pancreas, 3xUSP, cod. P1625) from Sigma–Aldrich (Buchs, Switzerland), ammonium bicarbonate (Carlo Erba, Italy), hydrochloric acid and hydrogen peroxide 30% (w/v) (Merck, Darmstadt, Germany) were used.

For cell culture and permeability assay (bioavailability), inactive fetal bovine serum, 2.5 g L^{-1} (w/v) trypsin sterile solution, 0.2 g L^{-1} (w/v) EDTA sterile solution (Cultilab, Campinas, Brazil), Dulbecco phosphate buffered saline (D-PBS) without Ca^{2+} and Mg^{2+} (Nutricell, Campinas, Brazil), non-essential amino acids (NEAA – 100x), 200 mmol L^{-1}(w/v) L-glutamine, 100 mmol L^{-1} (w/v) sodium pyruvate, 1 mol L^{-1} (w/v) HEPES, 100 UI mL^{-1} (w/v) penicillin and 100 µl mL^{-1} (w/v) streptomycin (Gibco, Life, Grand Island, USA), Dulbeccós modified Eaglés medium high glucose (DMEM, Sigma-Aldrich, St. Louis, EUA) were employed.

For tannins analysis, Folin-Denis reagent was prepared using sodium tungstate dihydrate, phosphomolybdic acid and phosphoric acid (Merck, Darmstadt, Germany) and sodium carbonate and tannic acid were purchased from Synth (Diadema, Brazil).

2.3. Analytical procedures

Trace elements were determined using an ICP OES (5100 VDV, Agilent Technologies, Tokyo, Japan) employing optimized conditions (Milani, Morgano, & Cadore, 2018): Radiofrequency power (RF), 1.35 kW; Ar flow rate, 12.0 L min^{-1}; Ar auxiliary flow rate, 0.50 L min^{-1}; seaspray nebulizer flow rate, 0.55 L min^{-1}; quartz double-pass spray chamber; axial plasma view; wavelengths: Al (396.152 nm), As (193.6906 nm), Cd (214.439 nm), Co (228.615 nm), Cr (267.716 nm), Cu (324.754 nm), Fe (259.940 nm), Li (670.783 nm), Mn (257.610 nm), Ni (221.648 nm), Pb (220.353 nm), Sb (206.834 nm), Se (196.026 nm), Sn (189.925 nm), Sr (421.552 nm) and Zn (213.857 nm).

2.3.1. In vitro bioaccessibility

In vitro bioaccessibility was evaluated using a static digestion method based on the works of Laparra, Vélez, Montoro, Barberá, and Farré (2003) and Peixoto, Devesa, Vélez, Cervera, and Cadore (2016), allowing the simulation of the gastrointestinal phases of the human digestion process. This procedure did not include an 'oral phase' ever since beverages residence in mouth is very short (Minekus et al., 2014). The bioaccessible concentration was calculated as a ratio, in percentage, among the contents of trace elements in bioaccessible fraction and in ready-to-drink ice tea.

For the gastric phase: 10 mL of ice tea samples were added in graduate tubes and the pH was adjusted to 2.0 using a selective electrode (Starter 3100, Ohaus, Barueri, Brazil) with 6 mol L^{-1}(v/v) HCl. Pepsin solution was added to provide a proportion of 0.02 g pepsin / 10 mL sample and the tubes were incubated in a water bath (Tecnal, Piracicaba, Brasil) at 37 °C for 2 h, under agitation.

For the intestinal phase: The pH of gastric extract was adjusted to 5.0 with 20% NH_4OH (w/v) and a pancreatin + bile extract solution was added to provide a proportion of 0.0050 g pancreatin + 0.030 bile extract / 10 mL sample. The tubes were incubated for two additional hours. Then, the pH of gastrointestinal extract was adjusted to 7.0 with few drops of 20% NH_4OH (w/v), tubes were refrigerated (4 °C) and the extracts were centrifuged (Fanem, São Paulo, Brazil) at 3500 rpm for 30 min. The supernatant was filtered through a PTFE 0.45 µm membrane and the resulting solution (bioaccessible fraction) was separated for further microwave digestion.

All analysis were performed in triplicate and considered blank experiments.

2.3.2. Microwave digestion

For trace elements analysis, samples were digested in a closed microwave digestion system (Start D, Milestone, Sorisole, Italy), using conditions described in our prior work (Milani et al., 2018): 2 mL of ice tea sample or 4 mL of bioaccessible extract were transferred to PTFE digestion vessel with 10 mL of diluted oxidant solution (5 mL purified water + 4 mL purified HNO_3 + 1 mL H_2O_2). Sample decomposition was performed at a maximum temperature of 120 °C for 43 min and the final solutions were transferred to 20 mL volumetric flasks using purified water.

2.3.3. Cell culture and permeability (bioavailability) assay

Cell culture and permeability (bioavailability) study was performed according to Peixoto (2015) and Do Nascimento da Silva & Cadore (2019) studies. For cell culture, Caco-2 cells from Rio de Janeiro Cell Bank - BCRJ (passage 49) were maintained in DMEM supplemented with 10% fetal bovine serum, 1% NEAA solution, 1% L-glutamine solution, 1% sodium pyruvate solution, 1% HEPES solution and 1% antibiotics solution. Cells were incubated at 37 °C under 5% CO_2 atmosphere (Sanyo, Moriguchi City, Japan). Medium was changed every two days and cells passed at 70% confluence. Caco-2 cells were seeded in a minimum density of 5×10^4 cells/cm^2 in 24 mm 6 well Transwell® plates with a pore size of 0.4 µm (Corning Inc., New York, USA) and experiments were carried out after the differentiation period (21 days). For bioavailability assay, osmolality of bioaccessible fraction was verified using semi-micro osmometer (K-7400, Knauer, Berlim, Germany) and adjusted to 310 ± 10 mOsm kg^{-1} using NaCl. An aliquot of 1.5 mL of bioaccessible fraction was added in the apical portion (retention) whereas 2.0 mL of a solution of 0.7 g L^{-1} (w/v) KCl, 0.35 g L^{-1} (w/v) $NaHCO_3$, 8.0 g L^{-1} (w/v) NaCl and 1.0 g L^{-1} (w/v) glucose was added in basolateral portion (transport). Transepithelial electric resistance (TEER) between apical and basolateral portion was monitored using Millicell®electrode resistance system (Millipore, Madrid, Spain) and the microbiologic contamination was evaluated using optical microscopy. The plates were incubated for 2 h and the contents of both portions were separated for further analysis. Bioavailability (uptake) was calculated as the sum of retention and transport.

2.3.4. Tannins analysis

For tannins analysis, 200 µL of ice tea samples were transferred to graduate tubes containing 10 mL of purified water. Then, 1.0 mL of Folin-Denis reagent and 2.0 mL of saturated sodium carbonate solution were added and the volume was made up to 20 mL with purified water. The solution was kept under rest for 30 min and filtered using quantitative filter. Absorbance measurements were made using a UV–VIS spectrophotometer (Cary 50, Varian, Melbourne, Australia) at 760 nm (AOAC, 2012). Analytical curve was prepared using tannic acid solution in 1.0–10 mg L^{-1}. All analysis were performed in triplicate and considered blank experiments. The results were expressed as "tannic acid equivalent".

2.4. Quality control and statistical analysis

For quality control, sample analysis was performed using an optimized method described in details in our prior work (Milani et al., 2018). Briefly, trace elements analysis was performed in triplicate and blank experiments followed the same procedure used for ice tea samples. Analytical methods were validated based on INMETRO recommendations (INMETRO, 2016). Limits of detection (LOD) and quantification (LOQ) were calculated as $LOD = 0 + t_{(n-1,\ 1-\alpha)}.s$ and $LOQ = 10.s$, being "s" = standard deviation of seven blank experiments and t = 3.143 (99% confidence level). The LOD and LOQ values ranged from 0.3 to 12.0 µg L^{-1} and 1.1 to 38.2 µg L^{-1}, respectively and these values were within Brazilian (Brazil, 2013) and MERCOSUR (2011) regulations. Linearity of the analytical curves was determined by the correlation coefficient (Pearson or "r") and obtained values were r > 0.9999.

Accuracy was verified using certified reference materials RM 8433 Corn Bran (NIST, Maryland, USA) and INCT-TL-1 Tea leaves (Institute of Nuclear Chemistry and Technology, Warszawa, Poland) and spiked experiments in three levels (Al, Fe, Mn: 50 μg L^{-1}, 500 μgL^{-1} and 5000 μg L^{-1}; others: 5 μg L^{-1}, 50 μg L^{-1} and 500 μg L^{-1}). All experiments were performed in triplicate. Recoveries ranging from 79 to 110% and from 81 to 118% were verified for certified reference materials and spiked experiments, respectively. Both values were in agreement with AOAC (2016): 75–120%. Precision (n = 7) was evaluated considering the coefficient of variation (CV, in percentage) and values were below 8% for all trace elements in ice tea assays.

Statistical one-way analysis of variance (ANOVA) and Tukey's test were performed using XLSTAT software (Addinsoft, Paris, France), at 95% of the confidence level. For statistical tests, values below LOQ were considered null. Principal component analysis (PCA) was executed using Pirouette software (Infometrix, Woodinville, WA, USA). Data was organized in a matrix (19 × 7), where lines and columns corresponded to the ice tea samples and trace elements with high effect in model, respectively. Auto escalated pre-processing was used and no samples were considered outliers (Mahalanobis distance).

2.5. Estimation of mineral dietary intake and risk assessment

The contribution for minerals dietary intake was estimated considering one ice tea glass (200 mL) and the Cu, Fe, Mn, Se and Zn mean levels. The results were compared to Brazilian dietary reference intake (Brazil, 2005) for 4–6 years old children (Cu = 440 μg, Fe = 6 mg, Mn = 1.5 mg, Se = 21 μg, Zn = 5.1 mg) and adults (Cu = 900 μg, Fe = 14 mg, Mn = 2.3 mg, Se = 34 μg, Zn = 7 mg).

The evaluation of the potential risk related to the intake of inorganic contaminants from ice tea consumption was calculated considered the daily intake of one ice tea glass (200 mL) by an adult (body weight, bw, 60 kg) and a child (bw = 15 kg, according to FAO, WHO (2011) and the mean Al and Sr levels found in ice tea. For Al, The Joint FAO/WHO Expert Committee on food additives (JECFA) established Provisional Tolerable Weekly Intake (PTWI) in 2 mg/kg bw (FAO/WHO, 2019) and for Sr, The US Agency for toxic substances and disease registry (ATSDR) established in 2 mg/kg/day the minimal risk level (MRL) (ATSDR, 2019).

3. Results and discussion

3.1. Trace elements contents in ready-to-drink ice tea

Table 2 describes the contents (mean, median and range) for trace elements in ready-to-drink ice tea samples from Brazil.

In general, ready-to-drink ice tea samples presented low levels of inorganic contaminants and all of samples were found within Brazilian and MERCOSUR thresholds: As (0.05 mg kg^{-1}), Cd (0.02 mg kg^{-1}), Pb (0.05 mg kg^{-1}) and Sn (150 mg kg^{-1}) (MERCOSUR, 2011; Brazil, 2013). Even though these regulations did not present a maximum limit for Li and Ni, these trace elements were found at low concentration, ranging from < 6.2 to 31 μg L^{-1}. For Co, Cr and Sb, non-quantifiable levels were observed in all samples.

In general, trace elements levels were similar in black, green and white ice tea, according to the ANOVA and Tukey's test at 95% of the confidence level. These types of ice tea are produced by infusion of *Camellia sinensis* leaves, with different harvesting time or after fermentation process (Preedy, 2013). For Al, Cu, Fe, Mn, Se, Sr and Zn, wide

Table 2
Trace elements content in ready-to-drink ice teas: mean, median and range (n = 3).

Element	Results (μg L^{-1})	Black ice tea (n = 4)	Green ice tea (n = 6)	Mate ice tea (n = 6)	White ice tea (n = 3)
Inorganic contaminants					
Al	Mean (range)	2620 (2389–2892)^{c}	2378 (1458–3504)^{bc}	658 (289–2094)^{a}	1103 (875–1279)^{ab}
	Median	2600	2179	392	1156
As	Mean (range)	<LOQ	<LOQ	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Cd	Mean (range)	<LOQ	0.7 (<LOQ-4.3)	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Li	Mean (range)	<LOQ	1.4 (<LOQ-8.5)	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Ni	Mean (range)	<LOQ	<LOQ	5.2 (<LOQ-31)	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Pb	Mean (range)	<LOQ	<LOQ	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Sb	Mean (range)	<LOQ	<LOQ	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Sn	Mean (range)	<LOQ	<LOQ	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Sr	Mean (range)	130 (114–150)^{bc}	92 (39–119)^{ab}	171 (113–219)^{c}	43 (39–50)^{a}
	Median	128	113	173	41
Minerals					
Co	Mean (range)	<LOQ	<LOQ	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Cr	Mean (range)	<LOQ	<LOQ	<LOQ	<LOQ
	Median	<LOQ	<LOQ	<LOQ	<LOQ
Cu	Mean (range)	10 (<LOQ-14)	11 (<LOQ-23)	5.7 (<LOQ-12)	4.7 (<LOQ-14)
	Median	12	10	5.5	<LOQ
Fe	Mean (range)	132 (78–193)^{b}	20 (<LOQ-48)^{a}	123 (59–225)^{b}	14 (<LOQ-27)^{a}
	Median	129	16	110	14
Mn	Mean (range)	1329 (1285–1399)^{a}	2604 (1164–3883)^{a}	5013 (1210–6709)^{b}	707 (677–731)^{a}
	Median	1315	2313	5669	713
Se	Mean (range)	60 (35–87)^{b}	14 (<LOQ-50)^{a}	58 (29–87)^{b}	<LOQ
	Median	60	<LOQ	57	<LOQ
Zn	Mean (range)	66 (46–83)^{a}	102 (48–183)^{a}	155 (46–235)^{a}	50 (32–83)^{a}
	Median	67	73	151	34

* LOQ: As = 38.2 μg L^{-1}, Cd = 3.8 μg L^{-1}, Li = 6.2 μg L^{-1}, Ni = 25.7 μg L^{-1}, Pb = 10.9 μg L^{-1}, Sb = 10.3 μg L^{-1}, Sn = 18.0 μg L^{-1}, Co = 9.0 μg L^{-1}, Cr = 10.9 μg L^{-1}, Cu = 7.9 μg L^{-1} and Se = 26.7 μg L^{-1}. For statistical tests, values < LOQ were considered null. Inorganic contaminants thresholds (Brazil, 2013; MERCOSUR, 2011): As (0.05 mg kg^{-1}), Cd (0.02 mg kg^{-1}), Pb (0.05 mg kg^{-1}) and Sn (150 mg kg^{-1}).

[a, b, c] Mean values between different columns with the same letter are not significantly different at p > 0.05, according to the Tukey's test.

ranges were observed. The highest Al, Fe and Se levels were found in black ice tea samples (2620 $\mu g\ L^{-1}$, 132 $\mu g\ L^{-1}$ and 60 $\mu g\ L^{-1}$, respectively) while mate tea presented the highest Mn, Sr and Zn levels (5013 $\mu g\ L^{-1}$, 171 $\mu g\ L^{-1}$ and 155 $\mu g\ L^{-1}$, respectively). For green and white ice tea presented the lowest trace elements levels – except for Cu in green ice tea samples, ranging from < 7.9 $\mu g\ L^{-1}$ to 23 $\mu g\ L^{-1}$.

In general, Mn and Fe levels from our study were similar to the study reported by Erdemir (2018) for black and green tea infusions, ranging between 963 and 1240 mg kg^{-1} and 92.8 and 242 mg kg^{-1}, respectively. Olivier et al. (2012) reported higher Al, Fe, Mn and Zn contents in black, green and mate tea, ranging from 67 to 478 mg kg^{-1}, 3.5 to 6.5 mg kg^{-1}, 73 to 781 mg kg^{-1} and 12 to 33 mg kg^{-1}, respectively.

In our previous studies, we evaluated some trace elements in black, green, white and mate in tea infusions prepared considering the manufactures recommended proportion of 1 bag (~1.5 g) for 200 mL cup (Milani et al., 2016; Milani et al., 2019). Similar contents in black, green and white tea infusions were observed for Zn (22 to 70 $\mu g\ L^{-1}$) and Cu (17 to 32 $\mu g\ L^{-1}$) whilst Al (163 $\mu g\ L^{-1}$), Cu (1.7 $\mu g\ L^{-1}$), Fe (16.8 $\mu g\ L^{-1}$), Mn (2312 $\mu g\ L^{-1}$) and Zn (70 $\mu g\ L^{-1}$) low levels were found in mate tea infusions. Some studies from Poland also reported data from black and green tea infusions. Pohl et al. (2020) reported high Al, Cu, Fe, Mn, Sr and Zn levels in green tea infusions: 1.74 $\mu g\ mL^{-1}$, 0.0492 $\mu g\ mL^{-1}$, 0.0326 $\mu g\ mL^{-1}$, 1.14 $\mu g\ mL^{-1}$, 0.0372 $\mu g\ mL^{-1}$ and 0.0803 $\mu g\ mL^{-1}$, respectively and Polenchońska, Dambiec, Klink, and Rudecki (2015)

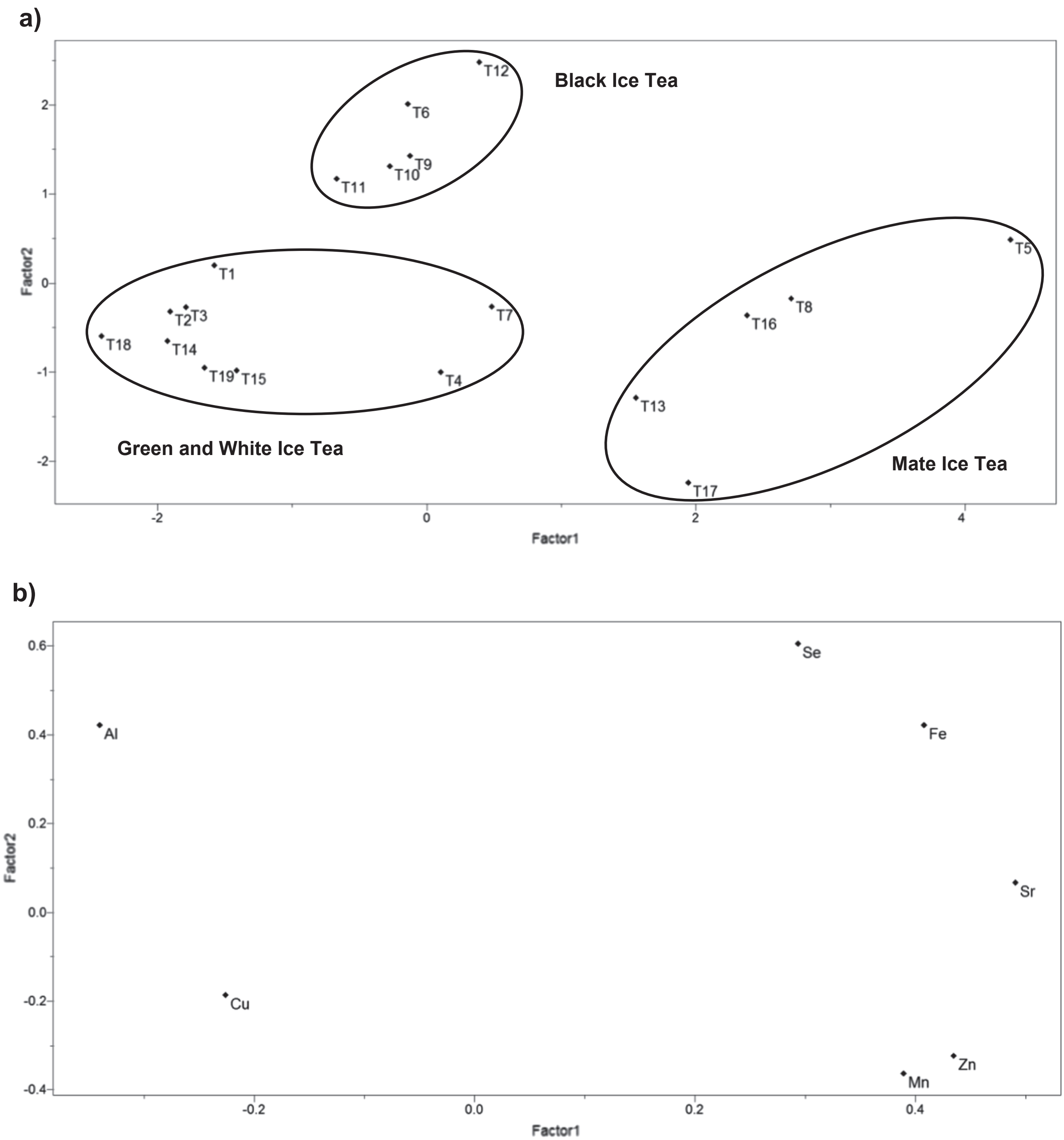


Fig. 1. Principal Component Analysis for trace elements in ready-to-drink ice tea: 1a) scores plot; 1b) loading plot.

found Al and Mn levels between 57 and 606 mg kg^{-1}, 60 and 136 mg kg^{-1}, respectively.

The differences verified between this study for ice tea and the ones for tea infusions may indicate different procedures adopted by those authors on tea infusions (up to 2 g per 100 mL) and the ready-to-drink ice tea industries. Another hypothesis is the tea processing, since high Cu, Cr, Fe and Ni levels were reported in tea samples after processing in stainless steel equipment (Zhang et al., 2018).

Principal components analysis (PCA) is a multivariate tool that provides interpretation for complex analytical data. For this study, trace elements with high effect in model (Al, Cu, Fe, Mn, Se, Sr and Zn) were considered and the results are present in Fig. 1.

Two principal components explained 70.57% of the total variance (Factor 1 = 49.63% and Factor 2 = 20.94%), being Factor 1 related to the trace elements Sr (0.4904), Zn (0.4347), Fe (0.4079), Mn (0.3889) and Al (-0.3402) whereas Factor 2 is related to Se (0.6060), Fe (0.4226), Al (0.4220) and Mn (-0.3636). The values in the parentheses correspond to the loading values. From Fig. 1, three groups were classified:

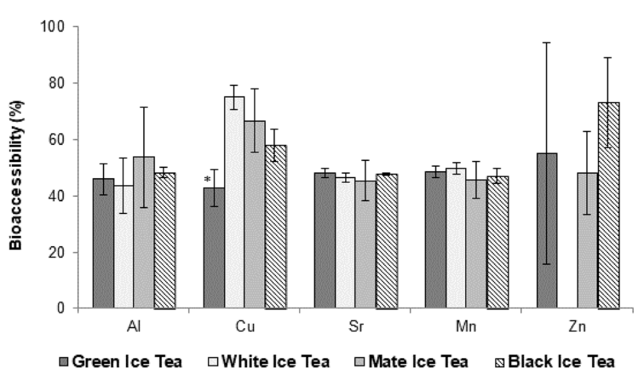
- Group 1 (mate ice tea): associated to Sr, Mn and Zn (positive loading in Factor 1);
- Group 2 (mainly black ice tea): associated to Al, Se and Fe (positive loading in Factor 1);
- Group 3 (green and white ice tea): associated to Cu (negative loading in Factor 1) and Al (positive loading in Factor 2).

Even though ready-to-drink ice tea samples considered different brands and flavors, multivariate analysis successfully categorized them into 3 groups based on similarities in their trace elements composition. The only exception was sample T6 (mate tea), which presented high Al level (2094 μg L^{-1}) and was categorized in group 2 (mainly black ice tea).

3.2. Trace elements bioaccessibility and bioavailability after in vitro digestion

The Al, Cu, Sr, Mn and Zn bioaccessible fractions in ready-to-drink ice tea were shown in Fig. 2. Levels below the limits of quantification were observed for the other trace elements.

In general, the bioaccessible fractions of Al, Sr, Mn and Zn in ready-to-drink ice tea corresponded to 50% of their total content. For Cu, low values were observed for green tea (37 to 50%), whilst mate and black tea presented high bioaccessible fractions (54 to 77%). These findings are in accordance to the reported interactions between trace elements (such as Al) and calcium, fluoride ions and polyphenolic compounds (such as tannins), both present in high levels in teas (Erdemoğlu, Pyrzyniska, & Güçer, 2000; Do Nascimento da Silva et al., 2015; Erdemir, 2018; Sanches et al., 2020). In addition, the presence of ingredients and flavors in ice tea may also influence trace elements bioaccessibility. Mate and black tea samples presented sugar in their composition (approximately 18 g / 200 mL), which has been reported as an enhancing factor in catechin bioavailability from tea (Peters, Green, Janle, & Ferruzzi, 2010).



* Mean values between bar with same pattern are significantly different at $p < 0.05$, according to Tukey's test. Error bars represent standard deviation.

Fig. 2. Al, Cu, Sr, Mn and Zn bioaccessibility from ready-to-drink ice tea.

For some ice teas, trace elements bioaccessibility variate widely, such as Zn in green tea (18% to 97%) and Al in mate tea (27 a 69%), feasibly due to differences in beverage manufacturing, formulation and the presence of ingredients or flavors. Schmite et al. (2019) reported significate variation in Al bioaccessibility in yerba mate tea added by ingredients such as lemon juice, cinnamon, honey and sugar. The authors verified values ranging from 0% (honey) to 91.3% (lemon juice) whilst the cold mate infusion presented Al bioaccessibility of 68 ± 11%.

Trace elements bioavailability experiments were performed using Caco-2 cells culture, which considerers trace element permeability through the cell monolayer (intact all through the test). In these experiments, the contents of apical (retention) and basolateral (transport) contents were measured. Trace element transport was calculated as a ratio between basolateral layer and bioaccessible contents, in percentage. Although all analytes presented slight variation in the apical layer concentration, only Sr and Mn were quantifiable in basolateral layer. The results are described in Table 3.

Transport ranged from ND to 6% and 2% to 4% for Sr and Mn, respectively. Manganese absorption has been reported to be small, being<2% of total content, especially in foods with high Zn level (Do Nascimento da Silva & Cadore, 2019). From Table 2, one could notice the same pattern between Mn bioaccessibility (Fig. 2) and bioavailability: green tea > black tea > mate tea. For Sr, novel information related to green and black tea was found: although these ice teas provided a similar Sr bioaccessibility, Sr transport in green ice tea were 50% higher than black tea.

It is important to mention that the low bioavailability values must be carefully interpreted. Cell culture model allows to measure the transcellular (through the cells) transport. Other possible transport pathways are the paracellular (between pores of adjacent cells) and the passive transcellular, the former highly influenced by the organic species present in matrix (Do Nascimento da Silva & Cadore, 2019). Trace elements in vegetables are reported to present more favorable interactions with organic species (such as polyphenols and tannins) than water (Do Nascimento da Silva et al., 2015) and, therefore, low transport values may be observed.

3.3. Tannins

Tannins were reported as the main compounds present in tea leaves (Preedy, 2013) and they were determined using Folin-Denis reagent, which produce a blue complex reacting with free and conjugated phenolic compounds, with maximum absorption between 620 and 765 nm (Granato et al., 2016). Method accuracy were verified by recovery experiments in three levels (1 mg L^{-1}, 4 mg L^{-1} and 5 mg L^{-1}) with results between 85 and 108%, in accordance with AOAC (2016) and INMETRO (2016) thresholds: 80 to 110% for 10 mg L^{-1} level. The mean tannins results for ice tea samples were 155 mg L^{-1}, 1041 mg L^{-1}, 982 mg L^{-1} and 702 mg L^{-1} for black, green, mate and white ice tea, respectively.

Trace elements bioaccessibility indicated a possible negative influence due to tannins presence in Cu bioaccessibility in green ice tea,

Table 3
Transport of Sr and Mn by Caco-2 cells (bioavailability, %).

Ice tea	Analyte	Bioaccessible (μg L^{-1})	Bioavailability Basolateral (μg L^{-1})	Transport (%)
Black tea (n = 3)	Sr	75 ± 5	2.80 ± 0.23	4
	Mn	652 ± 36	19.6 ± 1.7	3
Green tea (n = 3)	Sr	68 ± 1	4.00 ± 0.54	6
	Mn	254 ± 7	11.2 ± 2.1	4
Mate tea (n = 3)	Sr	98 ± 1	ND	–
	Mn	3424 ± 66	60 ± 21	2

***ND** = Not detected, [Sr] < 0.45 μg L^{-1}.

which presented the highest tannins contents. For Al, a pattern was observed for ice teas prepared using *Camellia sinensis* leaves: lower bioaccessible fractions were observed for white and green ice tea (44% and 46%, respectively) than black tea (48%), which presented the lowest tannins content.

For black ice tea, tannins levels were 10x lower than the green, white and mate tea. These levels are in agreement to the study reported by Konieczynski, Viapiana, and Wesolowski (2017), which verified higher levels of alkaloids (theobromine, theophylline and caffeine) in black tea than phenolic compounds. Green and mate tea were reported to present high levels of phenolic compounds, such as flavonoids and caffeic acid. In mate aqueous extracts (*Thea sinensis* extracts) high values can be found, ranging from 1.1 and 1.6% (Casanova & Arturo, 2012).

3.4. Mineral dietary intake and potential risk related to inorganic contaminants from ice tea consumption

The contribution for Cu, Fe, Mn, Se and Zn dietary intake from ice tea consumption was estimated considering their mean levels (Table 2). The results were shown in Table 4.

From Table 4, low contributions for Cu, Fe and Zn dietary intake were found considering the daily intake by adults and children (up to 1% DRI for Zn). On the other hand, ice tea was found to be Mn and Se source, contributing to 15% DRI, at least (Brazil, 2012). The highest Mn contribution were found in black, green and mate tea, ranging from 18 to 67% DRI for children and from 12 to 44% DRI for adults. For Se, the highest contribution to dietary intake was found for black and mate tea (57% and 35% for children and adults, respectively).

The results for potential risk assessment related to the intake of inorganic contaminants from ice tea intake by adults and children were presented in Table 5. Overall, the potential risk assessment revealed safe levels. For Sr, the daily consumption of one glass of mate ice tea may contribute up to 0.1% and 0.03% of PWTI for children and adults, respectively. For Al, higher contributions were observed: up to 3% of PTWI (green and black tea) for adults and 12% of PTWI (black tea) for children. Ever since this study considered only the potential contribution of a single dietary source, one could find higher values if the whole daily dietary were considered.

Nevertheless, recent studies suggest the importance to also consider the bioaccessibility and bioavailability of a trace element performing a risk assessment study (Villa, Peixoto, & Cadore, 2014; Do Nascimento da Silva & Cadore, 2019). In this present study, *in vitro* bioaccessibility evaluation demonstrated that the total content of trace elements in ready-to-drink ice tea may not correspond to the fraction available for absorption. Al bioaccessibility in the ready-to-drink ice teas, for example were proximate to 50% (Fig. 2), demonstrating the importance to perform this evaluation to an accurate potential risk assessment.

4. Conclusion

This study reported a comprehensive assessment of trace elements occurrence in ready-to-drink ice tea samples, including their total content, tannins and the influence of *in vitro* digestion in their bioaccessibility and bioavailability (using Caco-2 cells model). High levels were found for Al in black and green tea (2620 and 2378 μg L^{-1}, respectively), Mn in mate tea (5013 μg L^{-1}) and Sr in black and mate tea (130 and 171 μg L^{-1}, respectively). Multivariate analysis accurate classified ice tea samples in three different groups, based on their total trace elements content.

From bioaccessibility evaluation, Al, Cu, Sr, Mn and Zn bioaccessible fractions corresponded to, approximately, 40–60% of their total content in ready-to-drink ice tea samples. High tannins content were found in green and mate tea (1041 and 982 μg L^{-1}, respectively) and these results indicated a possible negative relation with trace elements bioaccessibility.

Mineral dietary intake and potential risk assessment considered two different scenarios: the daily consumption of one ice tea glass by adults and children. Although mate and black ice tea were found to be Mn and Se sources (dietary reference intake above 15%), the contribution for Al PTWI can reach 3.1% and 12% for adults and children, respectively. These findings indicate the importance on performing bioavailability studies and can contribute to the scientific community improvement of an accurate potential risk assessment.

Table 4
Dietary reference intake (DRI) assessment; considering the Cu, Fe, Mn, Se and Zn mean levels and the daily consumption of one glass of ready-to-drink ice tea (200 mL).

Ice Tea	Consumption	Trace elements				
		Cu	Fe	Mn	Se	Zn
Black	Intake (mg)	0.002	0.026	0.266	0.012	0.013
	% DRI (Children)	0	0	18	57	0
	% DRI (Adults)	0	0	12	35	0
Green	Intake (mg)	0.002	0.004	0.521	0.003	0.020
	% DRI (Children)	0	0	35	14	0
	% DRI (Adults)	0	0	23	9	0
Mate	Intake (mg)	0.001	0.025	1.003	0.012	0.031
	% DRI (Children)	0	0	67	57	1
	% DRI (Adults)	0	0	44	35	1
White	Intake (mg)	0.001	0.003	0.141	ND	0.010
	% DRI (Children)	0	0	9	–	0
	% DRI (Adults)	0	0	6	–	0

ND = Not Detected; **DRI** = Dietary Reference Intake for children: 4–6 years old (Cu = 440 μg, Fe = 6 mg, Mn = 1.5 mg, Se = 21 μg, Zn = 5.1 mg) and adults (Cu = 900 μg, Fe = 14 mg, Mn = 2.3 mg, Se = 34 μg, Zn = 7 mg) (Brazil, , 2005).

Table 5
Risk exposure assessment, considering the consumption of one glass of ready-to-drink ice tea (200 mL) and the Al and Sr mean levels.

Trace element	Consumption	Ice Tea			
		Black	Green	Mate	White
Aluminum	Weekly Intake (mg/kg bw)	0.061	0.055	0.015	0.026
	% PTWI (Adults)	3.1	2.8	0.8	1.3
	Weekly Intake (mg/kg bw)	0.245	0.222	0.061	0.103
	% PTWI (Children)	12	11	3.1	5.1
Strontium	Daily Intake (mg/kg bw)	0.0004	0.0003	0.0005	0.0001
	% MRL (Adults)	0.02	0.02	0.03	0.01
	Daily Intake (mg/kg bw)	0.002	0.001	0.002	0.001
	% MRL (Children)	0.1	0.05	0.1	0.05

PTWI: Provisional Tolerable Weekly Intake for Al = 2 mg/kg bw (body weight) (FAO/WHO, 2019); **MRL:** Minimal Risk Level for Sr = 2 mg/kg/day (ATSDR, 2019).

CRediT authorship contribution statement

Raquel Fernanda Milani: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Vitor Lacerda Sanches:** Formal analysis, Investigation, Methodology. **Marcelo Antonio Morgano:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Solange Cadore:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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

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