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Original Research Article

Bioavailability evaluation of calcium, magnesium and zinc in Brazilian cheese through a combined model of *in vitro* digestion and Caco-2 cells

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ABSTRACT

Cheese has important nutritional properties as a result of their protein, lipid and essential minerals content. This study analyzed the bioavailability of Ca, Mg and Zn in Brazilian cheeses, using the *in vitro* INFOGEST digestion method coupled with assays in Caco-2 cells. Furosine content (marker of the early Maillard reaction) was also evaluated. Two self-produced fresh cheeses, cow and goat *Minas frescal*; and two commercial matured goat cheeses, Blue and Pyramid, were analyzed. Mineral bioaccessibility after *in vitro* digestion was in the range of 44.4–74.7 %, 54.8–66.1 % and 18.2–38.7 % for Ca, Mg and Zn, respectively. Bioaccessibility was significantly affected by the mineral and fat content level. The digested fresh cheeses (*Minas frescal*) showed the highest values of soluble Ca and Mg; and the Pyramid goat cheese presented the greatest Ca and Zn transport efficacy across Caco-2 cell monolayers. Furosine could be a useful indicator to evaluate cheese ripeness and its indirect effect on the mineral availability.

1. Introduction

The European Mediterranean countries produce 2.5 million tons of goat milk annually, 95 % of which is destined for cheese production (Griffin et al., 2020). In recent years, cheese consumption in Brazil has increased by 2.7 % per year, when compared to the 2000–2010 period (FAOstat, 2018), with "Minas frescal" cheese being the dairy product most produced and marketed (IBGE, 2018).

The demand for goat cheese is related to its high digestibility and low calories, in comparison to cow's cheese which is less digestible and rich in cholesterol and other types of lipids (Haenlein and Anke, 2011). The consumption of goat cheese is also related to health maintenance and chronic disease prevention (Bergillos-Meca et al., 2015; Moreira et al., 2019). Cheese from different sources, including goat cheese, offers a high nutritional value for humans and is a rich source of nutrients that include proteins, lipids, vitamins and essential minerals. The main minerals reported in the composition of goat cheese are calcium (Ca), magnesium (Mg) and zinc (Zn) (Moreira et al., 2019).

During the digestion process, only a portion of the ingested minerals

is available for absorption and use in physiological functions, i.e., only a fraction of the total dietary mineral is bioavailable (Cardoso et al., 2015; Mesías et al., 2009). The availability of the minerals contained in the cheese can be affected by the type of technology used for production, the protein fraction, and fat content and quality (Khouzam et al., 2011). Technological aspects applied during the production of the different types of goat cheese (fresh, cured, matured) and the ripening process can considerably affect the digestibility levels of the minerals (Modzelewska-Kapituta et al., 2008; Silva et al., 2018).

Dietary minerals need to be released from the food matrix as a prerequisite for intestinal absorption, i.e., need to be bioaccessible. Bioaccessibility of minerals has been traditionally estimated through static methods of *in vitro* gastro-intestinal digestion; followed by determination of the mineral soluble fraction after centrifugation or dialysis through a membrane of a standardized pore size (Bosscher et al., 2003; Bergillos-Meca et al., 2015). However, pH, ionic strength, digestion time, the type and origin of the enzymes used in the digestion processes make it difficult to compare the results generated by different authors. For this reason, the COST-Action INFOGEST proposes a new method of

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static *in vitro* digestion; in order to harmonize and standardize the conditions of simulated digestion around the world (Minekus et al., 2014). This protocol is based on the physiological conditions found in the gastrointestinal tract and on previous determinations of real activity of the enzymes used in the digestive process (Brodtkorb et al., 2019).

To study the absorptive response following the digestive process, a combined model of *in vitro* digestion and human intestinal cultured cells using the Caco-2 cell line, may be successfully applied (Borges et al., 2015). These cells differentiate into mature enterocytes under suitable conditions, developing microvilli and acting in a similar way to small intestine epithelial cells; thus, being useful for estimating the transport and absorption of mineral elements (Alegría-Torán et al., 2015).

During the cheese-making process, technological operations such as heat treatment, homogenization, and pressure and coagulation can affect the structure of the milk constituents (Santiago-López et al., 2018). Heat treatment and ripening of cheeses also lead to the development of Maillard reaction products (MRP), which are nonenzymatic browning compounds produced when amino acids or proteins react with carbohydrates. Among these is furosine, which is generated from the acid hydrolysis of the Amadori compound lactuloselysine; it is considered a marker of the early stages of the reaction, and able to indirectly quantify the heat load applied during cheese production and degree of ripening (Schwietzke et al., 2011).

Due to the scarce information available in the scientific literature concerning this subject, this study aims to determine the bioavailability of essential minerals in different kinds of Brazilian cheeses, by applying a combined model of INFOGEST *in vitro* digestion and Caco-2 cells. In addition, furosine content was determined as a marker of the early Maillard reaction linked to protein quality and its possible relationship with mineral availability in cheeses analyzed.

2. Material and methods

2.1. Chemicals

All chemical reagents used for mineral and *in vitro* digestion analyses were procured from Sigma-Aldrich (St Louis, USA) and of analytical grade.

2.2. Samples

Four different kinds of Brazilian cheese were selected for analysis. Two were produced in a pilot plant (one sample was a dairy cow cheese and was used for comparison against goat cheese) and two samples were commercial goat cheeses.

Cheese obtained from the pilot plant was *Minas frescal*, a popular fresh type of cheese in Brazil. Cheese production was performed by obtaining samples of goat and cow's milk *in natura* form directly from two producers; located in Rio Claro-SP and Amparo-SP in Brazil, on three different days. The milk samples were heat treated by slow pasteurization (65 °C/30 min). After this process, the milk was cooled to 4 °C and stored in a cold chamber (4 ± 1 °C) until the cheese was processed. The efficiency of pasteurization was evaluated by alkaline phosphatase (AOAC, 2006; Method 979.13) and peroxidase (Lanara, 1981) enzyme activity.

The *Minas frescal* goat and cow cheese were prepared according to Diamantino et al. (2014), with modifications. The milk was heated to 35 °C, with 250 µg/mL of calcium chloride 50 %, previously activated (30 °C / 8 h) lactic culture (1.5 %, v/v) consisting of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (R704 - Chr. Hansen, Hoersholm, Denmark) and a coagulant (CHY-MAX Powder Extra NB - Chr. Hansen, Hoersholm, Denmark) in sufficient quantity to coagulate the milk in 35 min. The gel was cut, with the aid of horizontal and vertical liras, into cubes of 1.5–2.0 cm. After resting for 5 min, slow stirring was performed for 30 min. The curd was then kept at rest for 10 min and partial draining of the curd was started. A saline solution (1.3 % NaCl in

relation to the volume of milk) at 35 °C was added to the mass, followed by stirring and another rest period (10 min). The curd was placed in plastic molds and successive turnings were performed after 15, 30 and 45 min. The cheeses were fermented for 4 h at room temperature and stored in a cold chamber (4 ± 1 °C). After 24 h of refrigerated storage, the cheeses were removed from the plastic molds and their pH was measured to assure that fermentation was adequate.

In addition to the fresh self-produced cheeses, commercial Blue goat cheese and Pyramid goat cheese were purchased directly from a producer in Amparo-SP (Brazil), from three distinct batches. The Blue goat cheese is a Brazilian cured cheese inspired by blue Stilton English cheese, so called because of its veins of *Penicillium roqueforti* fungus. The Pyramid goat cheese is lactic-fermented and takes approximately 24 days to mature with charcoal, until its flowery bark is completed with white molds of the *Penicillium candidum* type.

Samples were freeze-dried at -40 °C for 48 h (lyophilizer model LS3000, Terroni, Brazil), vacuum packed and sent to the Department of Physiology and Biochemistry of Animal Nutrition, CSIC (Granada, Spain), where they were kept under refrigeration (4 ± 1 °C) until laboratory analyses were performed.

2.3. Chemical characterization of cheese: nutrient composition and mineral analysis

Analyses were performed in triplicate. Moisture (method 934.01) and total ash content (method 942.05) were determined using official methods (AOAC, 2000). Fat content was extracted using chloroform: methanol 2:1 and quantified by Soxhlet (AOAC, 2000). Total nitrogen was determined according to the Dumas procedure using LECO Truspec CN equipment (LECO Corporation, St. Joseph, USA). Protein content was calculated using the factor of 6.38. Gross energy of samples was determined in an isoperibolic bomb calorimeter (Parr Instrument Co., Moline, USA).

For the mineral analysis, all glassware and polyethylene sample bottles were washed with 10 mol/L nitric acid and demineralized water (MilliQ Ultrapure Water System; Millipore Corp., Bedford, USA) and the procedure described by Haro et al. (2020) was followed. Briefly, aliquots of ground freeze-dried cheese were wet digested with the addition of concentrated HNO₃:HClO₄ (1:4) and heating to high temperatures (180–220 °C) in a sand beaker (Block Digestor Selecta S-509; J. P. Selecta, Barcelona, Spain). Determination of Ca, Mg and Zn were carried out by flame atomic absorption spectroscopy (FAAS) in a Perkin-Elmer AAnalyst 700 Spectrophotometer (Norwalk, CT, USA). Total phosphorus was determined colorimetrically at 820 nm in a spectrophotometer (Shimadzu UV-1700, Model TCC-240A, Columbia, USA) by the vanadomolybdate procedure (AOAC, 2000; Method 965.17). Blank samples were included in order to decrease or eliminate the interference between different samples and chemicals used. Standard solutions were prepared from Tritisol (Merck, Darmstadt, Germany) and lanthanum chloride (0.3 %) was added to the samples and standards for Ca and Mg measurements, to avoid interferences. Certified external standards (European Commission, Reference Materials Unit, Geel, Belgium) were used to test the accuracy of the method: skimmed milk powder (ERM-BD150) for Ca and Mg, lyophilized brown bread (BCR191) for Zn and rye flour (ERM-BC381) for P. The measured values were always within the certified ranges. Analytical conditions and certified reference materials used are shown in Table 1.

2.4. *In vitro* digestion and bioaccessibility of minerals

In vitro digestion of the freeze-dried samples was performed in triplicate according to the INFOGEST protocol described by Egger et al. (2016), with some modifications. The procedure was composed of an oral, gastric and intestinal phase; however, due to the specific composition of cheese, salivary amylase enzyme was excluded in the simulated oral phase. Briefly, 0.5 g of freeze-dried sample was mixed with 4.5 mL

Table 1

Analytical conditions and certified reference materials of the mineral analysis.

	Analytical technique	λ nm	Certified reference standard	Certified value*	Measured value*
Ca	FAAS	422.7	ERM-BD150, Skimmed milk powder	13.9 \pm 0.8	13.7 \pm 0.4
Mg	FAAS	285.2	ERM-BD150, Skimmed milk powder	1.26 \pm 0.10	1.30 \pm 0.03
Zn	FAAS	213.9	BCR-191, brown bread	19.5 \pm 0.5	19.4 \pm 0.7
P	MAS	820.0	ERM-BC381, Rye flour	2.01 \pm 0.07	2.00 \pm 0.09

FAAS: Flame atomic absorption spectroscopy; MAS: molecular absorption spectroscopy.

ERM® Certified reference materials (ERM® is a registered trademark of the EC).

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* Ca, Mg and P concentration is expressed in g/kg. Zn concentration is expressed in mg/kg.

of MilliQ water to make a paste-like consistency. To replace the subsequent addition of the costly lipase enzyme in the intestinal phase, the samples at this point were sonicated (Vibracell VCX 130; Sonics & Materials INC, Danbury, USA) to facilitate the accessibility of lipids during subsequent steps of the digestion protocol as established by [Seiquer et al. \(2015\)](#). Next, 5 mL of simulated salivary fluid with 25 μ L of CaCl_2 was added to the samples and incubated at 37 °C for 2 min with mild and constant agitation. Then, 10 mL of simulated gastric fluid with pepsin and 5 μ L of CaCl_2 were added and the pH was lowered to 3.0 by adding 1 mol/L HCl (pepsin concentration on the final mix adjusted to 2000 U/mL). The mix was then incubated at 37 °C for 2 h with mild and constant agitation. Finally, 20 mL of simulated intestinal fluid with pancreatin, bile salts and 40 μ L of CaCl_2 were added and the pH was raised to 7.0 with 1 M NaOH (pancreatine concentration in the final mix adjusted to 13.37 mg/mL, while bile salts were adjusted to 10 mM), after which the mix was incubated at 37 °C for 2 h with mild and constant agitation. Then, the enzymatic reactions were immediately halted by freezing the tubes in liquid nitrogen. The frozen samples (containing pellet and digestion supernatant) were stored at -20 °C until soluble mineral analysis. For soluble mineral determination, samples were defrosted and centrifuged for 45 min, at 4000 rpm (Eppendorf 5810/R centrifuge; Merck, Darmstadt, Germany) and 4 °C. The bioaccessible fraction (BF) and the residual fraction (RF) were carefully separated and submitted to wet digestion as previously described, to determine the mineral content by FAAS. Blanks of the *in vitro* digestion were prepared with 5 mL of MilliQ water instead and the complete protocol was developed to detract the minerals present in the different fluids.

2.5. *Caco-2* cell experiments

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University (Spain). Culture flasks were purchased from Corning Costar (Cambridge, USA). The cells were maintained by serial passage in 75 cm² plastic flasks containing high-glucose Dulbecco's modified minimal essential medium (DMEM), with heat-inactivated fetal bovine serum (FBS) (10 %), NaHCO_3 (3.7 g/L), nonessential amino acids (1 %), HEPES (15 mM), bovine insulin (0.1 UI/mL), and 1 % antibiotic-antimycotic solution. The cells were grown under an atmosphere of air/ CO_2 (95:5) at 90 % humidity and 37 °C and given fresh medium every 3 days.

To evaluate mineral transport and uptake, cells were trypsinized and seeded into polycarbonate permeable supports (Transwell, 24 mm diameter, 4.7 cm² area, 3 μ m pore size, Costar) at a density of 40×10^4 cells/insert, with 2.5 mL of medium in the basal chamber and 1.5 mL in the apical chamber ([Seiquer et al., 2015](#)). The medium was changed every two days and cells were used for transport and uptake experiments after 20–21 days of culture, when cells were fully differentiated. Cell monolayer integrity during differentiation of Caco-2 cells was assessed by measuring the passage of phenol red across the monolayer, expressed as apparent permeability, according to [Ruiz-Roca et al. \(2008\)](#). Cell monolayers were used for absorption study when the leakage rate of phenol red was lower than 4 % per hour.

To ensure cell viability, BF was purified through ultrafiltration with a

cut-off membrane of 30 kDa to extract added digestion enzymes and macromolecular compounds (Amicon Ultra-15; Millipore, Darmstadt, Germany). Then, BF was used for the Caco-2 experiments diluted with 1:2 (v:v) DMEM without FBS; since cell viability after 2 h of exposure to purified samples under such conditions, assessed by the MTT assay, was never <85–90 %. On the day of the experiments, spent culture medium was aspirated from the apical and basolateral chambers and both cell surfaces were washed with Hank's balanced salt solution (HBSS) at 37 °C. Then 2.5 mL of the transport buffer Mg-free (130 mmol/L NaCl, 10 mmol/L KCl, 5 mmol/L glucose, and 50 mmol/L HEPES, pH 7) was added to the basolateral chamber, and the diluted BF of each cheese was added to the apical chamber (1.5 mL). After an incubation period of 2 h at 37 °C, the medium from the apical compartment was aspirated, the filter insert was removed, and the cell surface was washed with ice-cold buffer (150 mM NaCl, 1 mM EDTA, and 10 mM HEPES, pH 7) to remove nonspecifically bound metal and residual medium. The membrane with the cell monolayer was cut out and reserved to determine the mineral internalized in cells. To calculate the transport across the cell monolayer, the buffer from the basolateral chamber was removed and wells were washed with deionized water to ensure complete collection. The concentration of Ca, Mg and Zn was measured in the buffer from basal chambers and the cell monolayers by FAAS, as previously described.

Results of absorption and uptake of Ca, Mg and Zn were expressed as μ g/well and as percentage from the experimental solution added to the apical chamber. Since mineral availability is affected by solubility after digestion, the transport and uptake efficiency were expressed by considering differences in solubility, as follows:

Efficiency (%) = (% soluble mineral \times % transported or uptake mineral)/100.

2.6. Ion-pairing HPLC determination of furosine

Furosine was determined as described by [Delgado-Andrade et al. \(2007a,b\)](#), with minor modifications. Briefly, 50 mg of the lyophilized cheese samples were hydrolyzed with 4 mL of 7.95 M HCl at 110 °C for 23 h in a Pyrex screwcap vial with PTFE-faced septa. Hydrolysis tubes were sealed under nitrogen. The hydrolysates were aerated and cooled at room temperature and subsequently centrifuged at 14,000 \times g for 10 min. A 0.5 mL portion of the supernatant was applied to a Sep-pak Plus C₁₈ cartridge prewetted with 5 mL of methanol and 10 mL of deionized water and then eluted with 3 mL of 3 mol/L HCl. Samples were dried in a speedvac concentrator (Thermo scientific ISS110, Waltham, USA) for 2 h 30 min at 65 °C and dissolved in 1 mL of 0.2 % formic acid. The mobile phase was prepared with 5 mM sodium heptane sulphonate including 20 % of acetonitrile and 0.2 % of formic acid. An Extrasyl-ODS2 analytical column (25 \times 0.40 cm, 5- μ m particle size, Tecknokra, Barcelona, Spain) was used at 35 °C. The elution was isocratic and flow rate was 1.0 mL/min. The injection volume was 20 μ L and detection at 280 nm (diode array detector). Furosine was quantified by the external standard method. Calibration curves were built from a stock solution (1.2 mg/mL of furosine) in the ranges 0.1–10.0 mg/L and in 0.2 % formic acid. Analysis was conducted with a Shimadzu HPLC system (Kyoto, Japan) equipped with an LC-20AD pump, an SIL-10ADvp autosampler, a

CTO-10ASVP oven and an SPD-M20A diode array detector. The limit of quantification (LOQ) was set at 5 mg/kg. Analyses were done in duplicate and results were expressed as mg/kg and mg/100 g protein.

2.7. Statistical analysis

The data obtained was subjected to analysis of variance (one-way ANOVA), with cheese type as the main factor. The LSD test was used to compare mean values between cheeses, and differences were established at $P < 0.05$. The relationships between the different variables were evaluated by Pearson's coefficient. The StatGraphics Centurion XV software (StatPoint Technologies, Inc. USA, 2010) was used to carry out the comparisons.

3. Results and discussion

3.1. Chemical characterization of cheese: nutrient composition and mineral analysis

Table 2 shows the chemical composition of Brazilian cheeses. All the cheeses analyzed in this study had moisture percentages higher than 55 %, with the highest value for *Minas frescal* cow cheese ($P < 0.05$); however, the four samples were in a tight range of 55.6–56.6 %. Therefore, the analyzed cheeses may be classified as having high humidity, taking into consideration the guidelines of ordinances n° 146/96 and n° 352/97 that regulate the technical terms of identity and quality of Brazilian cheeses (Brazil, 1996, 1997). Chemical composition of the *Minas frescal* cheese was similar to that reported previously by Diamantino et al. (2014), although higher contents of ash (3.8 vs. 2.5 %) and protein (19 vs. 14 %) were found in the present study. Statistical differences ($P < 0.05$) were observed between samples in all chemical parameters analyzed (Table 1). Variations of 1.83–3.84 % for ash, 18.3–21.0 % for proteins, 19.6–21.1 % for fats and 298–324 Kcal/100 g for energy, are reported. According to Aljewicz and Cichosz (2015), these nutritional differences can be attributed to the chemical composition of the raw milk (cow and goat) and the production methods used in cheese production. These same factors were also referred to by Kira and Maihara (2007), who also indicated the season and cheese manufacturing location as being responsible for intense fluctuations in the nutritional composition. As the fat percentage of these Brazilian cheeses was lower than 24.9 %, they were classified as lean cheeses (10–24.9 %) (Brazil, 1996). Similar results were reported by Marques et al. (2020), who found maximum fat levels of 20.6 % in samples of fresh cheeses obtained from pasteurized and unpasteurized milk. Likewise, a recent study performed in dairy products reported 20.2 % fat matter content in fresh cheese with a moisture content of 61.2 %

(Hernández-Olivas et al., 2020), similar to the results of the present trial.

The mineral content (mg/100 g) of Brazilian cheeses is shown in Table 3. *Minas frescal* goat cheese was significantly richer in Ca and Mg ($P < 0.05$) in comparison to the others, although its Zn content was lower. The goat and cow *Minas frescal* and the Blue goat cheese showed a similar pattern in the concentration of analyzed minerals; with a predominance for Ca, followed by P, Mg and, finally, Zn content. Similarly, the Ca/P ratio was very close within these three kinds of cheese, with a mean value of around 1.50. In contrast, the Pyramid goat cheese exhibited a different pattern, as the P concentration was higher than the respective Ca value (218 vs. 103 mg/100 g, respectively); which led to the inversion of the Ca/P ratio and was significantly reduced in this sample ($P < 0.001$). Ca/P may be an important determinant of Ca absorption due to the regulatory mechanisms which control Ca and P homeostasis (Bass and Chan, 2006), and Ca/P values between 1:1 and 2:1 in a diet are considered positive for Ca balance and bone health (Loughrill et al., 2016). As mentioned, for the other nutritional parameters, several factors such as milk composition, manufacturing process across the cheese-making season, location (Gulati et al., 2019; To et al., 2020) or cheese slurry acidity during production (Aljewicz and Cichosz, 2015), may affect the mineral content of cheese; and ultimately, its biochemical and functional properties (Fox et al., 2017). For these reasons, extensive variability in the mineral profile of cheeses is reported within scientific literature. Aljewicz and Cichosz (2015) evaluated matured Dutch and Swiss cheese and reported amounts of Ca (903–980 mg/100 g) and P (551–579 mg/100 g) higher than the Brazilian cheeses in the present study. This was also the case for Hernández-Olivas et al. (2020), who reported Ca levels of 1260 and 1410 mg/100 g for fresh and aged cow cheese from Spain. In contrast, Modzelewska-Kapituła et al. (2008) reported mean Ca and P concentrations in white Polish cheese below those determined in the present study (97 and 99 mg/100 g, respectively), with a Ca/P ratio around 1. Closer to our data, Ünal et al. (2005) established a wide range of Ca levels, between 183.5–651.0 mg/100 g, in five different varieties of Turkish cheese. Concerning Mg, mean concentrations detected in this trial were within values previously documented in processed cheese (19.9 mg/100 g) (Crujisen et al., 2019); in Dutch and Swiss-type cheese (33.0 mg/100 g) (Aljewicz and Cichosz, 2015); and below levels of several cottage cheeses from the Slovak market (5.8–8.4 mg/100 g) (Capcarova et al., 2020).

Since milk is not considered a good dietary source of Zn, few investigations report Zn concentration in cheese. The former study by Capcarova et al. (2020) detected low Zn values of 0.18–0.23 mg/100 g. The higher moisture content of the cottage cheese compared with the short-ripened cheeses of the present assay could partially explain the high differences in the Zn and Mg content. On their part, Herman-Lara

Table 2
Nutrient composition of Brazilian cheese.

Type of cheese	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Energy (Kcal/100 g)
<i>Minas frescal</i> goat	55.6 ± 0.1 ^c	3.84 ± 0.1 ^a	19.7 ± 0.04 ^b	19.7 ± 0.05 ^c	298 ± 0.5 ^d
<i>Minas frescal</i> cow	56.6 ± 0.01 ^a	3.78 ± 0.03 ^a	18.3 ± 0.02 ^d	21.1 ± 0.03 ^b	302 ± 0.4 ^c
Blue goat cheese	55.9 ± 0.03 ^b	2.58 ± 0.03 ^b	21.0 ± 0.04 ^a	19.6 ± 0.1 ^c	315 ± 0.4 ^b
Pyramid goat cheese	55.7 ± 0.03 ^c	1.83 ± 0.03 ^c	19.5 ± 0.07 ^c	22.9 ± 0.1 ^a	324 ± 0.4 ^a

Data are mean value ± SE, expressed in a fresh matter basis. Different superscripts in the same column indicate significant differences ($P < 0.05$), ANOVA + LSD test.

Table 3
Mineral composition of Brazilian cheese (mg/100 g).

Type of cheese	Ca	Mg	Zn	P	Ratio Ca/P
<i>Minas frescal</i> goat	598 ± 14 ^a	41.6 ± 1.3 ^a	9.79 ± 0.2 ^b	387 ± 2 ^a	1.54 ± 0.06 ^a
<i>Minas frescal</i> cow	535 ± 9 ^b	37.6 ± 0.2 ^b	13.2 ± 0.07 ^a	348 ± 2 ^b	1.54 ± 0.03 ^a
Blue goat cheese	562 ± 5 ^b	32.7 ± 0.8 ^c	10.1 ± 1 ^b	389 ± 9 ^a	1.44 ± 0.05 ^a
Pyramid goat cheese	103 ± 2 ^c	13.6 ± 0.3 ^d	11.9 ± 0.1 ^a	218 ± 4 ^c	0.47 ± 0.01 ^b

Data are mean value ± SE, expressed in a fresh matter basis. Different superscripts in the same column indicate significant differences ($P < 0.05$), ANOVA + LSD test.

et al. (2019) reported Zn levels of 1.90 and 1.54 mg/100 g for fresh and mature artisanal Mexican goat cheeses, while Aljewicz and Cichosz (2015) detected Zn concentrations of 3.6–4.1 mg/100 g in their study. Therefore, in light of existing scientific literature, consumption of these Brazilian cheeses could be recommended for the daily intake of Ca, P and Mg (which are common elements in dairy products), and also for an additional supply of Zn.

On the other hand, the Ca, Mg and P content were strongly correlated in the studied cheeses ($r = 0.97$, $P < 0.001$), but no correlation with Zn was found (Supplementary Table S1). It was also observed that cheeses with the highest levels of Ca, Mg and P had the lowest content of fat ($r = -0.931$, -0.845 and -0.970 respectively, $P < 0.001$) and energy ($P < 0.001$).

3.2. In vitro digestion and mineral bioaccessibility

The *in vitro* gastrointestinal digestion method is a good approach to measure mineral bioaccessibility, but may not be an accurate reflection of actual bioavailability *in vivo*; however, it may be a useful indication of its potential absorbability by the intestine (Fardet et al., 2019). The bioaccessibility of Ca, Mg and Zn after the *in vitro* digestion method proposed by INFOGEST for samples of Brazilian cheeses is given in Table 4. Results were expressed as mineral solubility ($\mu\text{g/g}$) and bioaccessibility (percentage of soluble fraction from the initial quantity).

Analyzing the Ca values, Minas frescal goat and cow samples and Blue goat's cheese, which had similar Ca concentration of 562–598 mg/100 g, showed the highest soluble Ca after digestion; with a clear decrease in the case of Blue goat cheese (2666, 2777 and 1770 $\mu\text{g/g}$, respectively). Macronutrient composition does not seem to explain this difference. Therefore, other components such as casein phosphopeptides (CPP) (Etcheverry et al., 2012) or matrix factor, including initial texture of cheese, could be implicated in the final bioaccessibility of Ca (Fardet et al., 2019). It is also worth mentioning that the lowest calcium recovery, after the *in vitro* digestion, was in this sample (76.1 %).

The Pyramid goat cheese exhibited the least Ca content and soluble Ca (103 mg/100 g and 768 $\mu\text{g/g}$, respectively), but the highest percentage of bioaccessibility (74.7 %, $P < 0.05$), when compared to the other cheeses. This would suggest that the amount of Ca present in the cheese could be a limiting factor for its own solubility; in fact, Ca content in the samples were positively correlated with total solubility ($r = 0.869$), but inversely related to percentage of bioaccessible Ca ($r = -0.859$; $P < 0.001$) (Table S2). According to Fardet et al. (2019), Ca levels influence the structure of the cheese, giving rise to more cohesive and compact cheeses where the diffusion of the enzyme is restricted, therefore leading to reduced bioaccessible Ca.

Different investigations have studied Ca bioaccessibility in cheese. Values of 38.4 % for parmesan (Erba et al., 2017), 15 % for Dutch and

Swiss cheeses (Aljewicz and Cichosz, 2015), and a range of 11–16 % for fresh and aged cheese samples (Hernández-Olivas et al., 2020) have been reported. Based on Ünal et al. (2005), a small rate of Ca bioavailability (after dialysis) is the consequence of a high P/Ca ratio. In this sense, the high P content found in the samples, except for the Pyramid goat cheese, could lead to the formation of insoluble Ca-Mg-P complexes (Brink et al., 1995); a fact supported by the finding of an inverse P content-Ca bioaccessibility relationship ($r = -0.925$; $P < 0.001$, Table S2).

Other researchers have pointed to the content and type of fat, as well as to the fatty acid profile, as factors influencing Ca availability (Aljewicz and Cichosz, 2015). Accordingly, it was observed that the fat content was negatively related to the amount of soluble Ca ($\mu\text{g/g}$) ($r = -0.654$; $P < 0.05$) but positively associated with Ca bioaccessibility ($r = 0.948$, $P < 0.001$) in the Brazilian cheeses (Table S2). The effect of fat content on Ca solubility has been previously studied in processed cheese products and interesting results have been reported: increasing the fat content significantly reduced the water-soluble Ca. Although, in contrast, when expressed as a percentage of total initial Ca, the Ca solubility increases significantly with the fat content (Guinee and O'Callaghan, 2013); similar to the current assay. A negative effect on Ca availability is attributed to the formation of insoluble Ca - saturated fatty acid complexes, which are able to bind up to 60 % of ionized Ca (Bronner and Pansu, 1999). Unfortunately, the lipid profile was not analyzed, but only one of the cheeses was made from cow milk (Minas frescal), with a theoretically higher saturated fatty acid content. However, Ca bioaccessibility in this sample was greater than others made of goat milk, except the Pyramid goat cheese.

Levels of BF and percentages of bioaccessibility for Mg in Brazilian cheeses varied from 75 to 251 $\mu\text{g/g}$ and 54.8–66.1 %, respectively. Mg presented a different behaviour from that reported by Ca, since the initial Mg concentration in the samples was directly correlated with the total amount of soluble Mg ($r = 0.969$, $P < 0.001$) and with its bioaccessibility ($r = 0.677$, $P < 0.05$) (Table S2). Minas frescal cow cheese exhibited significantly higher percentages of bioaccessible Mg, closely followed by Minas frescal goat and Blue goat cheese. According to the lowest Mg content and solubility after *in vitro* digestion, Pyramid goat cheese showed the smallest percentage of Mg BF ($P < 0.05$). Similar to our findings, the study by Aljewicz and Cichosz (2015) reported an average availability of Mg around 64 % in the analyzed cheeses. Contrary to the case of Ca, the high P concentration of the samples did not seem to depress the bioaccessibility of Mg (total amount nor %BF), as good correlations were shown with the P level ($r = 0.908$, $P < 0.001$; $r = 0.743$, $P < 0.01$, respectively). Of course, other components apart from minerals should contribute to variations observed in divalent cation availability (Ca and Mg), as the generation of chelate bonds between metal ions, free amino acids, dipeptides and tripeptides produced during

Table 4
Bioaccessibility of Ca, Mg and Zn after *in vitro* digestion of Brazilian cheese.

Mineral	Type of cheese	BF ($\mu\text{g/g}$)	RF ($\mu\text{g/g}$)	% BF	% RF	Recuperation (%)
Ca	Minas frescal goat	2666 \pm 19 ^b	2524 \pm 60 ^a	44.4 \pm 1.1 ^c	42.2 \pm 2 ^a	86.7 \pm 3.7 ^b
	Minas frescal cow	2777 \pm 19 ^a	1823 \pm 51 ^b	51.9 \pm 1.2 ^b	34.1 \pm 1 ^b	85.9 \pm 1.7 ^b
	Blue goat cheese	1770 \pm 17 ^c	2504 \pm 42 ^a	31.5 \pm 0.3 ^d	44.6 \pm 1.1 ^a	76.1 \pm 1.1 ^c
	Pyramid goat cheese	768 \pm 9 ^d	252 \pm 7 ^c	74.7 \pm 1.8 ^a	24.6 \pm 1 ^c	99.3 \pm 2.6 ^a
Mg	Minas frescal goat	251 \pm 8 ^a	110 \pm 6 ^a	60.4 \pm 1 ^b	26.4 \pm 1.2 ^c	86.8 \pm 1.6 ^b
	Minas frescal cow	248 \pm 2 ^a	114 \pm 1 ^a	66.1 \pm 0.5 ^a	30.3 \pm 0.3 ^b	96.4 \pm 0.5 ^a
	Blue goat cheese	199 \pm 5 ^b	112 \pm 0.5 ^a	60.9 \pm 2.3 ^b	34.3 \pm 0.8 ^a	95.2 \pm 2.9 ^a
	Pyramid goat cheese	75 \pm 2 ^c	38 \pm 1 ^b	54.8 \pm 0.8 ^c	28.1 \pm 1.3 ^{bc}	82.8 \pm 1.3 ^b
Zn	Minas frescal goat	37.9 \pm 2.2 ^a	28.8 \pm 1.5 ^c	38.7 \pm 2 ^a	29.4 \pm 1.7 ^b	68.1 \pm 2.3 ^b
	Minas frescal cow	24.1 \pm 4.1 ^b	70.4 \pm 6.4 ^a	18.2 \pm 3.1 ^b	53.2 \pm 4.6 ^a	71.4 \pm 5.3 ^{ab}
	Blue goat cheese	30.3 \pm 2.8 ^{ab}	54.3 \pm 1.7 ^b	30 \pm 4.3 ^a	53.7 \pm 7 ^a	83.7 \pm 10.6 ^a
	Pyramid goat cheese	25.0 \pm 2.6 ^b	58.5 \pm 2 ^b	20.9 \pm 2.1 ^b	45.6 \pm 1.6 ^a	66.5 \pm 3.1 ^b

BF, Bioaccessible fraction; RF, residual fraction. Different superscripts in the same column indicate significant differences between samples for Ca, Mg or Zn ($P < 0.05$), ANOVA + LSD test.

digestion has already been described (Henry and Benz, 1995).

On the other hand, similar to Ca, the fat content seemed to influence the Mg bioaccessibility of cheese. In this case, inverse correlations with Mg solubility after digestion were found, both for absolute and relative values ($r = -0.817$, $P < 0.01$; $r = -0.627$, $P < 0.05$, respectively). Scarce information is found in scientific literature concerning Mg bioaccessibility in cheese, but more investigations have been developed for other dairy products. Recent research has established Mg solubility after digestion for infant formulas and milks greater than 60 % (Fioravanti et al., 2020); or even above 70 % in whole, semi-skimmed and skimmed UHT milks (Lacerda Sanches et al., 2020). Trials performed in young and adult rats have shown that Mg absorption is lower in cheese than for other dairy products (Delisle et al., 1995).

Concerning Zn, Brazilian cheese showed percentages of bioaccessible Zn ranging from 18.2–38.7 % (*Minas frescal* goat being the stand out). In general, for Ca and Mg, the residual fraction (RF) showed values lower than their respective bioaccessible percentages. However, the opposite was observed for Zn, whose recovery was also lesser than the former minerals, varying between 66.5 and 83.7 %. These overall low levels of bioaccessible Zn could be due to the fact that in dairy products most of the Zn is linked to casein, and it should be released at the acid pH of gastric digestion. However, a considerable proportion of this protein is not digested and under duodenum conditions (where Zn would be absorbed), the mineral might not be in free form to be taken up. The release of Zn binding phosphopeptides resulting from the trypsin and chymotrypsin activities on caseins could also affect Zn solubility (Pabón and Lönnnerdal, 2000). The initial Zn content of the cheese was negatively associated with its solubility after the digestive process, both when expressed as total value and as a percentage from total Zn ($r = -0.83$, $P < 0.001$). Therefore, the higher the Zn content in cheese (*Minas frescal* cow and Pyramid goat cheese), the lower the bioaccessible fraction ($P < 0.05$). On the other hand, an inverse correlation was again found between the fat content and the bioaccessibility of Zn ($r = -0.585$, $P < 0.05$) (Table S2); suggesting a negative influence of this nutrient on Zn solubility. Very few studies deal with the bioaccessibility of Zn in cheeses. Aljewicz and Cichosz (2015) established Zn availability from ripened Dutch-type cheese at ~61 % and from ripened Swiss-type cheese at ~70 %, far from those measured in the present study. These researchers observed a marked increase in Zn availability during the ripening and storage of cheeses, operations that were not controlled in our experimental conditions. Zn solubility after *in vitro* digestion of cheese pizza has been found to be 17.4 % (Singh et al., 2016), a similar result to that observed in the present study. Higher values have been reported in other dairy products, above 80 % in UHT milks (Fioravanti et al., 2020) and above 60 % in whole, semi-skimmed and skimmed UHT milks (Lacerda Sanches et al., 2020).

No correlations were found between mineral availability and the

protein content of the cheeses studied.

3.3. Transport and uptake of Ca, Mg and Zn from the BF of cheeses in Caco-2 cells

The bioaccessible fraction is not always fully absorbed by the intestine epithelium; therefore, to better determine mineral availability, the Caco-2 cell model is frequently coupled to the *in vitro* digestion process (Bergillos-Meca et al., 2015). The results obtained in the assays for transport and uptake of Ca, Mg and Zn in differentiated Caco-2 cells for Brazilian cheese samples are summarized in Table 5. The calculated efficiency of transport and uptake (taking into account solubility after digestion) is depicted in Fig. 1.

Percentage of Ca absorbed across epithelial cell monolayers (% transport) ranged between 5.30 and 7.25 %, with the highest value corresponding to *Minas frescal* goat cheese, which in contrast presented the lowest percentage of Ca uptake. When data was expressed as transport and uptake efficiency, Pyramid goat cheese showed significantly higher values than the other samples. There are 2 pathways through which Ca may be absorbed from gut lumen: transcellular and paracellular. Cell uptake represents the first step in transcellular absorption, although both concepts are not totally equivalent. Among factors affecting Ca bioavailability, fat has a positive impact in the direct absorption of Ca; and its contribution depends on the type of fatty acids (Wawrzyniak and Suliburska, 2021). Higher pH levels induced by a low-fat diet has been associated with significant reductions of Ca bioavailability, since a low pH reduces the formation of calcium phosphate, which leads to increased calcium absorption (Bandali et al., 2018). Accordingly, correlations between fat content and efficiency of Ca transport (a tendency, $r = 0.499$, $P = 0.098$) and uptake ($r = 0.866$, $P < 0.001$) (Table S2) in Caco-2 cells were found in the present study. This could explain the higher Ca availability in the Pyramid goat cheese, because of the higher fat content. In addition, this cheese had the lowest pH value after digestion among the studied samples (7.10 vs. 7.22, 7.32 and 7.30 in *Minas frescal* goat, *Minas frescal* cow and Blue goat cheese, respectively).

On the other hand, levels of Mg and P in the cheeses did not seem to affect Ca transport across the intestinal monolayer, but negative correlations were observed with uptake efficiency (Table S2); suggesting that the transcellular pathway could be affected. No statistical relationship was found between Ca availability and Zn content in the studied cheeses. Previous research in animals indicated that goat-milk cheese increases Ca absorption compared to bovine milk derived cheese. This is due to the high content of peptides from casein α_{s2} and β (Mora-Gutierrez et al., 2007), although in the present study no clear differences were observed due to the milk origin.

Regarding Mg, minor differences between samples were observed in

Table 5

Transport and uptake of Ca, Mg and Zn in Caco-2 cell monolayers after 2 h exposure to bioaccessible fractions obtained after *in vitro* digestion of Brazilian cheese.

Mineral	Type of cheese	Apical chamber ($\mu\text{g}/\text{well}$)	Transport ($\mu\text{g}/\text{well}$)	% Transport	Cell content ($\mu\text{g}/\text{well}$)	% Uptake
Ca	<i>Minas frescal</i> goat	51.4	3.73 ± 0.4^a	7.25 ± 0.8^a	1.37 ± 0.2^b	2.67 ± 0.3^b
	<i>Minas frescal</i> cow	48.8	2.74 ± 0.04^b	5.61 ± 0.1^b	1.68 ± 0.3^{ab}	3.44 ± 0.5^{ab}
	Blue goat cheese	49.8	2.92 ± 0.2^b	5.87 ± 0.5^{ab}	1.55 ± 0.2^{ab}	3.11 ± 0.5^{ab}
	Pyramid goat cheese	48.5	2.60 ± 0.2^b	5.30 ± 0.4^b	2.13 ± 0.2^a	4.39 ± 0.5^a
Mg	<i>Minas frescal</i> goat	30.1	1.71 ± 0.2^a	5.66 ± 0.7	2.10 ± 0.2	6.97 ± 0.7^c
	<i>Minas frescal</i> cow	24.4	1.15 ± 0.03^b	4.70 ± 0.1	2.06 ± 0.05	8.45 ± 0.2^b
	Blue goat cheese	22.1	1.11 ± 0.1^b	5.01 ± 0.5	2.10 ± 0.04	9.47 ± 0.2^b
	Pyramid goat cheese	19.2	0.92 ± 0.1^b	4.76 ± 0.4	2.23 ± 0.1	11.6 ± 0.6^a
Zn	<i>Minas frescal</i> goat	1.56	0.07 ± 0.01^a	4.60 ± 0.7^c	0.20 ± 0.03^a	12.9 ± 1.9^c
	<i>Minas frescal</i> cow	0.40	0.03 ± 0.01^c	8.82 ± 0.7^b	0.07 ± 0.01^b	16.1 ± 1.7^c
	Blue goat cheese	0.50	0.05 ± 0.01^b	10.7 ± 0.2^b	0.16 ± 0.01^a	32.9 ± 2.9^b
	Pyramid goat cheese	0.17	0.03 ± 0.01^c	20.3 ± 1.4^a	0.10 ± 0.01^b	58.4 ± 4.8^a

AC, apical chamber; BC, basal chamber. Different superscripts in the same column indicate significant differences between samples for Ca, Mg or Zn ($P < 0.05$), ANOVA + LSD test.

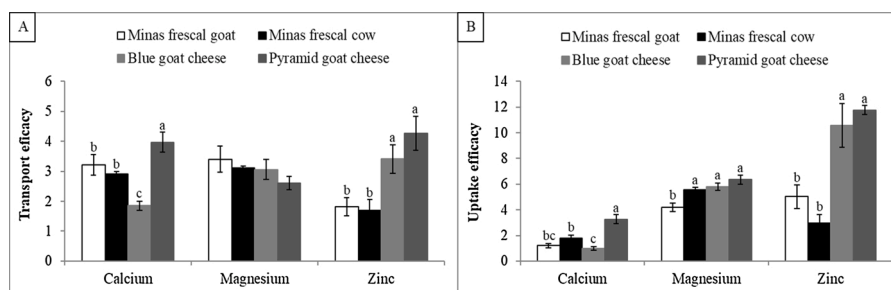


Fig. 1. Transport efficacy (A) and uptake efficacy (B) of Ca, Mg and Zn in Caco-2 cell monolayers after 2 h exposure to bioaccessible fractions obtained after the *in vitro* digestion of Brazilian cheese. Different letters indicate significant differences between samples for Ca, Mg or Zn ($P < 0.05$), ANOVA + LSD test.

transport values across Caco-2 cell monolayers, and not expressed as percentage from the initial amount or as efficiency. However, Mg uptake by cells significantly varied among cheeses, from 11.6 % for Pyramid goat cheese to 6.97 % for *Minas frescal* goat cheese. In a similar manner to Ca, two transport systems, one passive (paracellular) and one active (transcellular), are known to be responsible for Mg intestinal absorption. The solubility of Mg is an important factor, with increased solubility correlating to increased absorption (Workinger et al., 2018). In the present work we found that Mg content in cheese, in addition to being correlated to Mg solubility after digestion, was also positively related to Mg intestinal transport efficiency ($r = 0.773$, $P < 0.01$, Table S2). However, negative relationships were found with Mg cell uptake ($r = -0.612$, $P < 0.05$), thus suggesting that Mg content selectively favored the paracellular route. Compared with Ca, we observed similar values for transport percentages and higher values of Mg cell uptake than Ca. Previous studies in Caco-2 cells have shown that when Ca and Mg are presented at the same time, Mg received priority in absorption. This is due to the higher binding ability of Mg to CPP, a class of peptides derived from casein during digestion and that plays important roles in mineral absorption (Cao et al., 2017).

Concerning Zn, the Pyramid goat cheese showed the highest values of transport and uptake by Caco-2 cells, followed by Blue goat cheese and with both *Minas frescal* cheeses having the lowest values. Percentages of Zn uptake by intestinal cells were observed to be always considerably higher than those for transport; supporting that Zn kinetic absorption is a saturable process mainly governed by the uptake at the apical membrane of the intestinal mucosa (Maares and Haase, 2020). High percentages of Zn transport across the cell monolayers (up to 20 %) and extremely high percentages of uptake (reaching more than 50 %) were observed in the cheeses of the present study. However, due to the relatively low solubility after digestion, values for transport and uptake efficiency (Fig. 1) were drastically reduced, compared to percentage of soluble Zn transport (Table 4). Thus, although Zn content in the cheese was highly correlated with solubility after digestion, no relationships were found with transport or uptake efficiency by Caco-2 cells. This supports that Zn absorption does not only depend on an adequate dietary level, but is also greatly affected by its intestinal availability (Maares and Haase, 2020). Apart from the positive influence of dietary protein levels, the interrelation between Zn absorption and different micronutrients is still subject to ongoing research. Among factors affecting Zn availability in the present study, levels of Ca, Mg and P seemed to depress Zn absorption and uptake, while fat content of cheeses had a positive influence (Table S2).

The Caco-2 cell model coupled to the *in vitro* digestion has previously been used to study mineral availability in other dairy products, such as goat milk, fruit juices with whole or skimmed milk and dairy-based infant formulas (summarized in Bergillos-Meca et al., 2015). However, to the best of our knowledge, this is the first study concerning mineral availability from cheese in Caco-2 cells. It must be highlighted that, although Caco-2 cells are a useful tool for studying the mineral availability, the fractional absorption of *in vitro* cellular models is

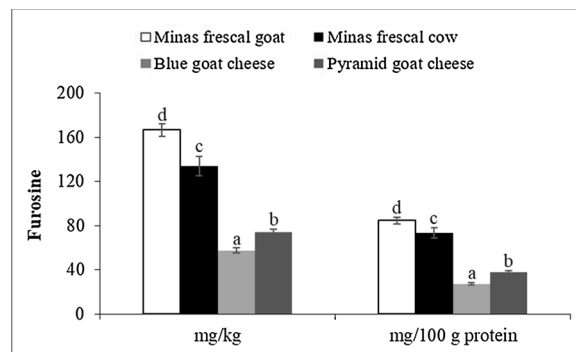


Fig. 2. Furosine content in cheese samples, expressed as mg/kg and mg/100 g protein. Different letters indicate significant differences between samples ($P < 0.05$), ANOVA + LSD test.

significantly lower than the estimated net absorption for humans *in vivo*. The discrepancy is mainly justified by differences in the absorption area ($1.1 - 4.7 \text{ cm}^2$ vs. about $30,000 \text{ cm}^2$) and ratios of volume/absorption areas, among others (Maares and Haase, 2020).

3.4. Furosine content of Brazilian cheese

Furosine content of Brazilian cheese ranged between 58 and 167 mg/kg ($27 - 85 \text{ mg/100 g protein}$), Blue goat cheese and Pyramid goat cheese showed significantly lower values compared to *Minas frescal* samples (Fig. 2). The decline in furosine content in these samples could be linked to the advancement of the Maillard reaction during ripening, and in turn lower the presence of the early compounds (Schwietzke et al., 2011; Spanneberg et al., 2012). The presence of the fungus *Penicillium*, has also been demonstrated to have fructosyl-amino acid oxidases that are able to catalyze the oxidative deglycation of fructosyl-amino acids, decreasing the Amadori compound and consequently the furosine concentration (Akazawa et al., 2004).

A significant inverse correlation was found between furosine and Zn availability (Table S3). In addition, Mg was sensitive to the furosine content, which could be related to the effect of Maillard reaction products on Mg bioavailability (Delgado-Andrade et al., 2007a,b).

4. Conclusions

The consumption of Brazilian cheese analyzed in the present assay may represent an essential contribution to the daily recommended intake of Ca, P and Mg, and provide a novel supply of Zn.

The initial mineral content and fat level affected mineral bioaccessibility after the *in vitro* digestion of Brazilian cheese. Fresh cheese showed the highest values of soluble Ca and Mg, and the matured Pyramid goat cheese showed the highest transport efficiency of Ca and Zn across the monolayers of Caco-2 cells. No correlations were found between the availability of minerals and the protein content of cheeses.

Furosine is a useful indicator to evaluate the ripening of cheeses and its indirect effect on mineral availability.

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José Luan da Paixão Teixeira: Methodology, Formal analysis, Writing - original draft. **Juliana Azevedo Lima Pallone:** Resources, Conceptualization, Writing - original draft, Supervision. **Cristina Delgado Andrade:** Methodology, Formal analysis, Investigation, Writing - original draft. **Marta Mesías:** Methodology, Formal analysis. **Isabel Seiquer:** Conceptualization, Investigation, Writing - original draft, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jfca.2021.104365>.

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