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Characterization and biological activity of ultrafiltrate goat whey protein concentrate over the *in vitro* digestion

Karla Kaligia Silva Borba [a], Carlos Gadelha [b], Katherine Gutiérrez-Álzate [c,d], Luciano da Silva Pinto [b], Marta Sueli Madruga [a], Maria Teresa Bertoldo Pacheco [e], Renata Maria Teixeira Duarte [f], Rita de Cássia Ramos do Egypto Queiroga [a,1], Marion Pereira da Costa [c,d], Marciane Magnani [a,*], Tatiane Santi Gadelha [b]

[a] *Post-Graduation Program of Food Science and Technology (PPGCTA), Department of Food Engineering, Federal University of Paraíba, João Pessoa, PB, 58051-900, Brazil*
[b] *Department of Molecular Biology, Center for Exact Sciences and Nature, Federal University of Paraíba (UFPB), João Pessoa, PB, 58051-900, Brazil*
[c] *Veterinary Medicine and Zootechnics, Federal University of Bahia (UFBA), Ondina Street, Salvador, BA, 40170-110, Brazil*
[d] *Faculty of Pharmacy, Federal University of Bahia (UFBA), Barão de Jeremoabo Street, Salvador, BA, 40170-115, Brazil*
[e] *Center of Science and Food Quality, Institute of Food Technology (ITAL), Campinas, SP, 13070-178, Brazil*
[f] *Multidisciplinary Center for Chemical, Biological and Agricultural Research at the State University of Campinas (CPQBA), UNICAMP, Campinas, SP, CEP 13083-876, Brazil*

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ABSTRACT

This study aimed to characterize the peptide profile of a goat whey protein concentrate subjected to *in vitro* gastrointestinal digestion using pepsin and pancreatin enzymes and evaluate the resulting hydrolysates' antioxidant and antimicrobial properties. The whey protein concentrate was obtained through ultrafiltration and analyzed across three stages: undigested (ND), gastric digested (GD), and gastrointestinal digested (GID). Molecular size and protein composition were assessed using SDS-PAGE/Tricine electrophoresis. Hydrophobicity was evaluated via column chromatography, and free amino acid profiles were determined. Antioxidant activity was measured using $DPPH^{-+}$ and $ABTS^{-+}$ assays. In contrast, antimicrobial activity was assessed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration against foodborne pathogens, spoilage bacteria and yeast. The results revealed that α-lactoalbumin was degraded in the GD phase, whereas β-lactoglobulin showed significant degradation after pancreatin action in the GID phase. High molecular weight (>52 kDa) whey proteins formed diffuse bands after enzymatic action. Chromatographic analysis showed peptide release in the GID phase and a reduction of peaks in goat whey (3.42 ND to 0.34 GID). The GID phase exhibited a significant increase ($p < 0.05$) in the release of essential (56%–80.9%) and branched chain (6%–15%) amino acids. GID peptides showed the highest antioxidant activities with 64.68% ($ABTS^{-+}$) and 47.95% ($DPPH^{-+}$) inhibition and the strongest activity against *Escherichia coli* and *Listeria innocua*. These findings underscore the bioactive potential of goat whey protein hydrolysates following simulated gastrointestinal digestion, highlighting their promise as functional ingredients with antioxidant and antimicrobial properties.

1. Introduction

Goat milk is recognized for its health benefits, which include improved digestion and gastrointestinal function due to its unique fatty acid composition, smaller fat globules, and low αs1-casein content. These characteristics reduce its allergenic potential, enhance mineral absorption, and promote desirable dietary intake (Nayik et al., 2022). Goat's milk is widely used in cheese production in various countries, generating a significant amount of whey. Goat whey is a valuable resource, rich in proteins of high biological value, such as

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* Corresponding author.
E-mail address: magnani2@gmail.com (M. Magnani).

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immunoglobulin, lactoferrin, lactoperoxidase, serum albumin, casein, β-lactoglobulin, and α-lactoalbumin. These proteins possess an amino acid profile associated with health-promoting properties, including inhibition of malignant cell proliferation and significant antioxidant potential, presenting promising opportunities for innovation in the dairy industry (Campos et al., 2022; Nayik et al., 2022; Li et al., 2024).

Whey proteins, in the form of concentrates or isolates, have been highly demanded in the food industry due to their use in the production of dietary supplements, nutraceuticals, medicinal products, nutritional preparations, and functional ingredients (Samtiya et al., 2022; Tarchi et al., 2024). The interest in whey is based on their high biological value and the presence of bioactive peptides derived from milk proteins, which have protective effects on human health (Nielsen et al., 2024; Shivanna & Nataraj, 2020; Ulug et al., 2021). These bioactive peptides are generated mainly through enzymatic hydrolysis of whey or milk proteins, modifying their functional and nutritional properties. Due to its relevance, enzymatic hydrolysis has been the subject of several investigations, particularly in the study of their antioxidant (Espejo-Carpio et al., 2016; Cui et al., 2024), antimicrobial (Campos et al., 2022; Lestari et al., 2022), antihypertensive and anti-aging (Sakkas et al., 2022; Rama et al., 2024; Rodrigues et al., 2024) activities. In addition, beneficial effects have been observed in preventing and treating cardiovascular, hepatic, gastrointestinal, immune, and nervous system diseases (Zhao et al., 2022).

In vitro enzymatic hydrolysis process of whey proteins depends on factors such as enzyme used, enzyme-substrate ratio, pH, temperature, and time (Shivanna & Nataraj, 2020; Sakkas et al., 2022). This process is carried out through *in vitro* digestibility tests that simulate gastrointestinal conditions using enzymes such as pepsin (Gong et al., 2022), trypsin (Sakkas et al., 2022), papain (Lestari et al., 2022) and chymotrypsin (Rama et al., 2024), which decompose whey proteins into peptides and amino acids.

Although there is much research on bioactive peptides produced in goat milk or cow's whey protein hydrolysates, studies on goat whey protein concentrate are limited. In this study, the peptide profile of a goat whey protein concentrate subjected to *in vitro* gastrointestinal digestion using pepsin and pancreatin enzymes was comprehensively analyzed. The investigation extended to evaluate the antioxidant and antimicrobial properties of the hydrolysates, aiming to explore their potential as bioactive ingredients in functional foods and nutraceuticals. The carefully conducted experiments and detailed findings contribute to bridging the gap in the literature regarding goat whey bioactivity, offering insights relevant to food science and biotechnology.

2. Materials and methods

2.1. Material and reagents

The goat milk used in this study to obtain the whey came from goats of the Saanen x Alpina-Americana breed in the early lactation phase (approximately 45 days), raised under grazing and confinement conditions with a diet based on forage and concentrates. The animals were located in the municipality of São João do Cariri, in the northeastern region of Brazil in Paraíba state. The region has a warm semi-arid climate with an average annual rainfall of less than 600 mm. These details are provided to ensure reproducibility and contextualize the results, given the known influence of these factors on milk composition (Mellado et al., 2022; Zhao et al., 2023).

Enzymes used in the *in vitro* digestion (α-amylase, pepsin and pancreatin), bile salts, molecular and chromatographic standards, 2,2′-diphenyl-2-picrylhydrazyl hydrate (DPPH) and hydroxy-2,5,7,8-tetramethylchromane-2-carboxyl acid (Trolox) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals reagents used in experiments were obtained from Neon (São Paulo, Brazil).

2.2. Goat whey protein concentrate

The goat whey protein concentrate was obtained using the ultrafiltration technique, following the methodology described by Pavoni et al. (2020) with minor modifications. A Millipore Tangential Flow Membrane with a 10 kDa cut-off (PLCC 5K Regenerated Cellulose - P34490) was used, with a feed flow rate of 34 mL/min and a whey temperature of 45 °C ± 5 °C to concentrate the protein. For further purification, in diafiltration mode ultrafiltration was applied, using a volumetric concentration factor of 6, where ultrapure water was added to the concentrate at the same removal rate as the permeate. The concentrate was lyophilized after processing, and the total protein content was quantified using the micro-Kjeldhal method (AOAC, 2012).

2.3. In vitro gastrointestinal digestion of goat whey protein concentrate

The *in vitro* digestion of the goat whey protein concentrate was conducted using the method proposed by Alencar-Luciano et al. (2023) with modifications. The lyophilized concentrate was reconstituted in ultrapure water at 40 mg/mL and subjected, in six replicates, to two-phase gastric and gastrointestinal digestion.

For gastric digestion (GD), the pH was adjusted to 2.8 by adding 6 M HCL, followed by the addition of 0.5 mL of pepsin suspension (prepared in 0.1 mol/L HCL at 4 g/100 mL) (Neon Comercial Ltda 1:10000). Samples were incubated in a water bath with internal agitation at 37 °C for 2 h. Subsequently, a portion of the samples was centrifuged at 9000×*g* for 15 min at 4 °C to precipitate the insoluble high molecular weight compounds and enzymes, effectively stopping the digestion process. The soluble portion (lyophilized GD) was collected.

The digestion continued for gastrointestinal digest (GID) by adjusting the pH to 5.7 with 5 M NaOH. This pH value was selected based on previous research simulating the early duodenal environment, where gastric chyme begins the neutralization process but has not reached complete alkalinity (Alencar-Luciano et al., 2023; Gong et al., 2022). Moreover, this pH coincides with the optimal range for pancreatin activity, favoring efficient hydrolysis and release of bioactive peptides. A 2.5 mL of a mixture of pancreatin (porcine pancreatin P 1750 - Sigma) and bile salts (porcine bile extract B 8631 - Sigma-Aldrich) (0.2 and 1.2 g suspended in 100 mL of 0.1 mol/L NaHCO3) were added to the reaction medium. The samples were incubated in a water bath with internal agitation at 37 °C for 2 h. The digest was centrifuged (9000×*g* for 15 min at 4 °C), and the soluble gastrointestinal digest (lyophilized GID) was collected. Samples of undigested (ND), gastric-digested (GD), and gastrointestinal-digested (GID) materials were dialyzed using a peptide membrane (Spectrum™ Spectra/Por™ Biotech Cellulose Ester (CE) Dialysis Membrane Tubing, MWCO: 100–500 Da). Finally, samples were frozen and lyophilized at −40 °C (Haas et al., 2024).

2.4. Electrophoretic profile (SDS-PAGE/Tricine)

The goat whey hydrolysates concentrated were analyzed using SDS-PAGE/Tricine polyacrylamide gel electrophoresis. Samples were dissolved in 0.625 M Tris-HCl buffer, pH 6.7, containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue. These were heated at 100 °C for 10 min, centrifuged (9000×*g* at 4 °C) for 5 min, and aliquots of 15 μL were applied to the gel. A three-phase gel system was applied: stacking (4% T; 3% C), spacer (10% T; 3% C), and separation (16.5% T; 3% C). Electrophoresis was performed at 110 V, 25 mA, and 7 W. Gels were fixed, stained with Coomassie Brilliant Blue G-250, and destained using 10% acetic acid. A low molecular weight marker (3.5–38 kDa) (Amersham ECL Rainbow Molecular Full Range, GE Healthcare Life Sciences) was used for molecular weight determination (Borba et al., 2022).

2.5. Hydrophobicity profile

Samples were diluted in ultrapure water and homogenized in an Ultra-Turrax tube disperser (Ika, Staufen, Germany) for 10 min at 18,000 rpm. The solution was then placed in an ultrasonic bath for 10 min (Unique, 1400, São Paulo, Brazil) and centrifuged at 2060×*g* for 10 min at 4 °C. Sample supernatants were filtered through filter paper and transferred to a syringe containing a filter with pore diameters of 0.45 μm and then 0.20 μm. The hydrophobicity profile of the peptides was assessed by reverse-phase HPLC using a Nova-Pak C18 column (4.6 m × 250 mm, 4 μm particle size, cartridge, Waters, Ireland), coupled to a high-performance liquid chromatograph (Waters 2690, California, USA). 20 μL of the soluble extract was injected, using a linear gradient of eluent A (1% trifluoroacetic acid in ultrapure water) and eluent B (1% trifluoroacetic acid in acetonitrile) as the mobile phase for 60 min, at a flow rate of 1 mL/min. Detection was performed at 218 nm (de Queiroz et al., 2017).

2.6. Electrophoretic profile by capillary electrophoresis

Twenty-five mg of the samples (ND, GD, and GID) were weighed and transferred to 10 mL volumetric flasks, which were volumetrically filled with borate buffer solution pH 9.3, obtaining solutions of 2.5 g/L concentration. The solution was agitated and sonicated in a water bath for 2 min and filtered before transferring to a siliconized vial for injection into the capillary system. Electromigration was carried out using an HP-3D capillary electrophoresis system (Hewlett Packard). Separation was carried out using a 72 cm × 75 μm inner diameter fused silica-coated capillary (HP G1600-2332) and the sample was injected for 8 s at a pressure of 50 mbar. Proteins and peptides were separated at a constant voltage of 25 kV. The temperature was maintained at 30 °C and the protein components were detected by UV absorbance at 214 nm (Pacheco et al., 2002).

2.7. Free amino acid profile

The free amino acid profile was determined following the methodology described by Cavalcanti et al. (2021). Samples were hydrolyzed with 6 mol/L HCl for 24 h, after which free amino acids were quantified using multilevel internal calibration with methionine sulfone as an internal standard.

Deproteinization was achieved by mixing the samples with methanol acidified with 0.1 mol/L HCl in an 80:20 (MeOH:0.1 mol/L HCl) ratio, combined with the sample and internal standard at a 7:2:1 ratio. High-performance liquid chromatography (HPLC) analysis was performed using a system (Thermo Fisher Scientific Inc., Rockford, USA) equipped with a LUNA C18 column (100 Å pore size; 250 mm × 4.6 mm; 5 μm particle size; 00G4252-EQ) (Phenomenex, Torrance, CA, USA).

2.8. Antioxidant activity: DPPH and ABTS assays

The scavenging capacity of the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{-+}$) was determined by Espejo-Carpio et al. (2016), mixing 350 μL from each sample (0.05 g/mL) with 315 μL of $DPPH^{-+}$ solution (60 μmol/L) diluted in ethanol P.A. The mixture was stirred for 85 min in the absence of light and centrifuged at 14,500×*g* for 5 min at 4 °C. After centrifugation, the absorbance of the supernatant was measured at 517 nm. The concentration of the $DPPH^{-+}$ radical in the reaction samples was determined from a calibration curve using Trolox. Free radical scavenging capacity was expressed as the percentage inhibition of the $DPPH^{-+}$ radical oxidation.

For the ABTS assay, the ABTS•+ radical was prepared following da Cruz et al., 2020 adapted methodology. A stock solution was created by dissolving 0.192 g of ABTS•+ in 50 mL of distilled water, followed by the preparation of a working solution by mixing 5 mL of the stock with 88 μL of $K_2S_2O_8$, and diluting 1 mL of this mixture in 70 mL of ethanol to achieve an absorbance of 0.700–0.800 nm at 734 nm.

A standard curve was constructed using a Trolox solution. Each sample (350 μL, 0.05 g/mL) was combined with 315 μL of the ABTS•+ solution, homogenized, and reacted for 6 min before measuring absorbance at 734 nm. The free radical scavenging capacity was quantified as the percentage inhibition of radical oxidation and expressed using the Trolox-equivalent antioxidant capacity (TEAC), consistent with the method applied for the DPPH•+ assay.

2.9. Antimicrobial activity

The antimicrobial activity of the samples was determined by microdilution assay on sterile plates. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal or Fungicidal Concentration (MBC/MFC) were evaluated and expressed in mg/mL. The samples were tested against standard strains of the following microorganisms: *Escherichia coli* ATCC 11775, *Salmonella choleraesuis* ATCC 10708, *Bacillus subtilis* ATCC 5061, *Pseudomonas aeruginosa* ATCC 13388, *Staphylococcus aureus* ATCC 6538, *Listeria innocua* ATCC 33090 and *Candida albicans* ATCC 10231. The concentration range of the hydrolysates tested was 10 to 0.001 mg/mL. The procedures followed the guidelines established in Methods for antimicrobial susceptibility testing of anaerobic bacteria (CLSI, 2022).

2.10. Statistical analysis

All analyses were done in three distinct experiments in triplicate. The analysis of variance (ANOVA) was performed to evaluate the data of the whey protein concentrate chromatograms (undigested, gastric digested, and gastrointestinal digested), the amount of free amino acids generated during *in vitro* digestion, and the bioactivity. Mean ± standard deviation was compared using Tukey's test, with a significance level set at $p < 0.05$. The analyses were carried out using the Statistical Analysis System software (SAS, 2002).

3. Results and discussion

3.1. Electrophoretic profile (SDS-PAGE/Tricine)

In SDS-PAGE/Tricine electrophoresis (Fig. 1), it was possible to evaluate the average molecular weight of the proteins in the undigested samples, as well as the changes of the band sizes corresponding to the same proteins during the complete gastric and gastrointestinal phases, showing the efficiency of the enzymatic hydrolysis on the proteins and, consequently, the release of peptides. It also indicates the presence of intact proteins resistant to digestion.

The protein profile of goat milk whey concentrate, presented in Fig. 1, was analyzed by SDS-PAGE using a molecular weight standard – std (3.5–38 kDa) as a reference to estimate the sizes of proteins and peptides in the samples. This analysis allowed the identification of key proteins present in the whey, such as β-lactoglobulin (β-Lg) (17 kDa) and α-lactoalbumin (α-La) (11 kDa) and immunoglobulins (Ig) (24–38 kDa), whose concentrations were lower than those observed in the study by Campos et al. (2022) and Xiao et al. (2023) on goat whey crude protein extract and goat whey proteins, respectively, which identified immunoglobulins (Ig), lactoferrin (LF), serum albumin (AS), β-lactoglobulin (>18 kDa) and α-lactoalbumin (>14 kDa). This difference can be attributed to factors related to the diet, breed, stage of lactation, and health of the goats from which the whey is sourced (Borba et al., 2022; Campos et al., 2022). This has a direct effect on specific protein concentrations. As well as possible effects of protein aggregation due to the sensitivity of protein to temperature and pH conditions, both the stages of sample processing and the ultrafiltration technique used to obtain the protein concentrate must be considered, which could affect protein retention and reduce the final percentage (Macedo et al., 2021).

The α-lactoalbumin that was present in the undigested concentrate

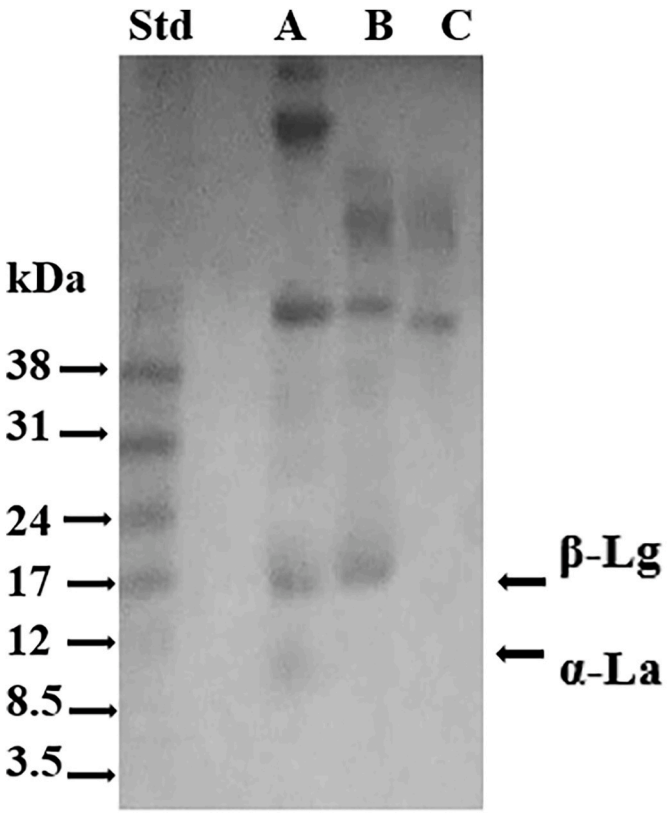

Fig. 1. Electrophoretic profile (SDS-PAGE/Tricine) of goat whey concentrate proteins: (Std): GE molecular marker (3.5–38 kDa); (A) Undigested; (B) Gastric digested; (C) Gastrointestinal digested.

(A) was no longer observed after gastric (B) and gastrointestinal (C) digestion, indicating that this protein was hydrolyzed by the enzymes pepsin and pancreatin (Fig. 1). In contrast, β-lactoglobulin remained intact on gastric digestion by the enzyme pepsin but was affected by pancreatin and was no longer detectable in the whey concentrate after gastrointestinal digestion. In contrast to the findings of Hettinga et al. (2023), who reported resistance of β-lactoglobulin from goat whey to pepsin and pancreatin, whereas α-La was hydrolyzed by the latter in gastric digestion at pH 4. Once, α-lactoalbumin becomes more susceptible to pepsin hydrolysis at pH < 4, which explains the rapid degradation. Therefore, the observed differences could be attributed to the pH values used in this study, which were 2.8 for digestion with pepsin and 5.7 for digestion with pancreatin, and to the resistance of β-lactoglobulin to digestion. Thus, hydrolysis of β-lactoglobulin may have important implications, as its degradation has been associated with a reduction in the allergenicity of this milk protein (Nayik et al., 2022).

It was also observed that high molecular weight proteins (>52 kDa) were hydrolyzed by pepsin during gastric digestion (Fig. 1). Similar results were reported by Xiao et al. (2023), who showed that proteins such as lactoferrin, serum albumin, and immunoglobulin G (IgG) heavy chain were degraded after gastrointestinal digestion. However, human milk proteins remained partially intact, indicating a higher resistance to gastric digestion than other milk sources. In Xiao et al. (2023) study, the gastric digestibility of total human milk protein was 48.89%, significantly higher than that of goat milk, which was 32.49%.

In Fig. 1, on the other hand, we could not detect smaller peptides, which could be related to the dialysis process used during sample preparation. Although this procedure effectively removes free amino acids and extremely small peptides using a predefined molecular weight cut-off limit (MWCO), it can also result in the loss of smaller peptides, including those with bioactive potential. Karami and Akbari-Adergani (2019) have highlighted that smaller peptides possess greater bioactive capacity, attributed to their high intestinal absorption rate and efficient entry into cells, compared to larger peptides. For their part, studies, such as those of Espejo-Carpio et al. (2016), have shown that peptides below 500 Da can sometimes be less bioactive than those within the range of 500–1500 Da, where antioxidant activity may be mainly affected depending on the composition and structural conformation of the peptide. This approach aligns with the objective of the study to retain peptides with higher bioactive potential.

Likewise, it is possible that the dialyzed samples contained low molecular weight peptides that were not visible by SDS-PAGE due to the limitations of its sensitivity to low molecular weight species and the low throughput of the membrane used (Xia et al., 2019). SDS-PAGE is more efficient in detecting proteins and peptides molecular weights above 3.5 kDa, as indicated by the molecular weight marker used. However, peptides smaller than this threshold may migrate too quickly and not be retained in the gel or may not stain effectively with Coomassie Brilliant Blue, which has reduced sensitivity for low molecular weight species (Sharma et al., 2021). Peptides between 3.5 and 10 kDa may still be present but could appear as faint, diffuse bands, which are less distinct compared to intact proteins. The peptides produced during digestion may have molecular weights below the lower detection limit of SDS-PAGE (e.g., <3.5 kDa). This would explain why only proteins above 38 kDa are visible. Post-digestion, the proteins above 38 kDa observed in the gel could represent undigested or partially digested fragments of whey proteins resistant to enzymatic hydrolysis. Therefore, the absence of visible peptides below 10 kDa in SDS-PAGE after digestion may result from limitations in the gel's resolving power and staining sensitivity for low molecular weight species. This reasoning is supported by Fig. 1 and by discussions of peptide detection limits in the literature (Borba et al., 2022), so that the absence may be due both to the size of the peptides, which would be below the detection range of the technique, and to the specific conditions for performing SDS-PAGE. In addition, bioactive peptides generated by protein hydrolysis during digestion play a crucial role in physiological functions and health promotion as immunomodulators, osteoprotectants, antimicrobials, anti-inflammatories, antioxidants, antihypertensives, hypocholesterolemics, anticarcinogens and antidiabetics (Nielsen et al., 2024). The bioactivity of these peptides is influenced by enzyme-substrate concentration, protein type, and reaction conditions such as pH and temperature (Shivanna & Nataraj, 2020; Samtiya et al., 2022). For instance, peptides derived from lactoferrin or immunoglobulins can exhibit antimicrobial, antioxidant, immunomodulatory, or antihypertensive properties, depending on the sequences released during hydrolysis (Campos et al., 2022).

Thus, the results reinforce the ability of pepsin to degrade complex proteins efficiently and highlight the potential of whey proteins as sources of bioactive peptides that could be used in functional or therapeutic applications. Recent studies, including those by Ma et al. (2021) and Hettinga et al. (2023), have investigated controlled enzymatic modifications to enhance the production of these peptides, aiming to optimize their bioactivity for use in food and pharmaceutical applications. Further studies should focus on this approach for goat whey hydrolysates.

3.2. *Hydrophobicity and electrophoretic profile of generated peptides and free amino acid profile*

The undigested (ND), gastric digested (GD), and gastrointestinal digested (GID) goat whey samples were characterized for their hydrophobicity of the peptides, electrophoretic profile, and free amino acid composition. The results reflect the specific action of the proteases used (pepsin and pancreatin), which catalyzed the release of peptides from the whey protein chains. Chromatographic analysis showed a significant decrease ($p < 0.05$) in the total peak area as digestion progressed, with goat whey concentrate being 3.42 in the undigested state and 0.34 in the gastrointestinal digested state (Table 1). This is attributed to the action of digestive enzymes that fragment complex molecules into smaller,

Table 1
Total area of chromatograms of goat whey protein concentrate during *in vitro* digestion.

	Digestion stages		
Goat whey protein concentrate	Undigested	Gastric Digested	Gastrointestinal Digested
	3.42 ± 0.33[a]	1.83 ± 0.07[b]	0.34 ± 0.01[c]

[a,b,c] Different letters on the same line differ statistically (Tukey test, $p < 0.05$).

more absorbable compounds such as peptides and free amino acids (Nielsen et al., 2024; Shivanna & Nataraj, 2020; Ulug et al., 2021).

Fig. 2 confirms the presence of peptides, evidencing an increase in lower-intensity peaks, attributable to the fragmentation of β-lactoglobulin and α-lactoalbumin into low molecular weight peptides after enzymatic hydrolysis. These fragments, probably smaller than 3.5 kDa, were not detected in the gel because of their possible migration outside the boundaries or to areas with limited detection, especially if the gel was overloaded. Although a 16.5% gel optimized for separating small proteins was used, it may not effectively retain such light peptides. Similar results have been reported by Cui et al. (2024), who observed similar differences in chromatographic profiles when analyzing the distribution of molecular weights in whey protein hydrolysates using different hydrolysis methods.

In the present study, peptides were separated by reversed-phase HPLC using a linear gradient of water (100–0%) for 60 min. Peptides eluted between 0 and 30 min showed hydrophilic properties, whereas those between 30 and 60 min showed hydrophobic properties. The elution profile obtained by reverse-phase HPLC reveals that all samples analyzed exhibited a predominantly hydrophilic peptide profile, reflecting the solubility characteristics of the peptides generated, which is a very important factor for those of proteins. This has important functional and technological implications from a biochemical and medicinal perspective for their application in liquid or semi-solid formulations (Qing et al., 2022).

Eberhardt et al. (2021) indicated that enzymatic hydrolysis can reduce the overall hydrophobicity of whey proteins, which is

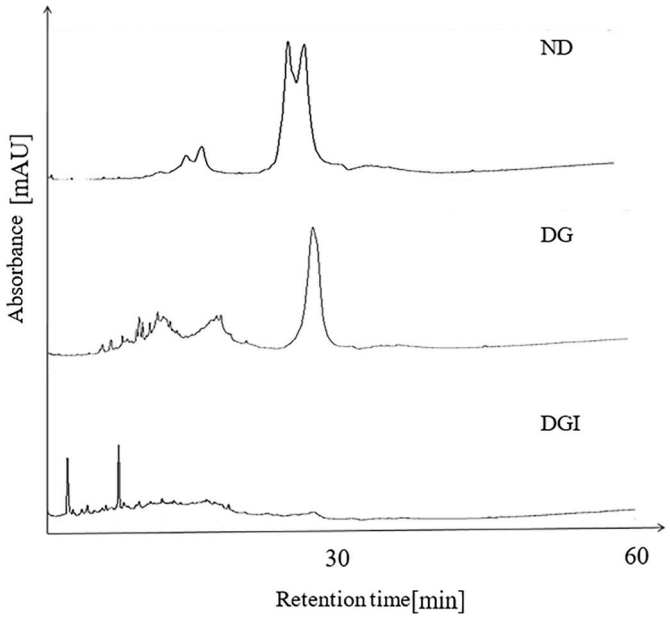

Fig. 2. Chromatogram of goat whey protein concentrate during *in vitro* digestion of samples ND = Undigested; GD = gastric digest; GID = gastrointestinal digest. [a,b,c] Different letters on the same line indicate a significant difference (Tukey's test, $p < 0.05$).

advantageous in food applications, as small hydrophobic peptides are often responsible for bitterness in protein hydrolysates. On the other hand, studies such as those by Rakesh et al. (2019) and Zhao et al. (2022) reported that peptides with polar residues exhibit antioxidant, antimicrobial, or immunomodulatory activities. These properties position them as promising candidates for developing functional foods and nutritional supplements, with potential applications in the adjunctive treatment of liver diseases, diabetes, sarcopenia, cancer, and cardiovascular diseases. In general, the hydrophilic profile observed in this study suggests that hydrolysis effectively released soluble peptide fragments, optimizing their absorption in the gastrointestinal tract, a crucial attribute for future nutraceutical applications. However, the limited presence of hydrophobic peptides could restrict specific functionalities, such as the ability to interact with lipids in emulsions or fatty matrices.

Capillary electrophoresis evidenced an increase in the number of peaks (peptides) when comparing ND, GD, and GID samples, confirming that as the degree of digestion increases, a greater amount of hydrolyzed species (polypeptides) present in the sample is generated (Fig. 3). This behavior is consistent with a progressive protein hydrolysis process, in which digestive enzymes act on intact proteins to release lower molecular weight fragments. Espejo-Carpio et al. (2016) observed that, during goat milk digestion, the molecular size distribution of the hydrolysates did not show significant changes after gastric digestion, suggesting that pepsin did not alter the originally generated hydrolysates. However, in the gastrointestinal phase, marked differences in the molecular profiles were evidenced due to the combined action of pancreatic enzymes and the initial releases of large protein fragments. This behavior supports the results of the present study, where the higher number of peptides detected in GID can be attributed to the synergistic effect of gastric and pancreatic proteases.

Simulated *in vitro* gastrointestinal digestion of milk whey induced the release of various amino acids, including those with functional and nutritional importance, such as essential threonine (Thr), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), lysine (Lys), histidine (His) and branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine. The increased concentration of total free amino acids in GD and GID compared to ND confirms the proteolytic effect of digestive enzymes. It highlights the efficiency of the simulated digestive system in protein degradation. Where free amino acid profile analysis (Fig. 4) revealed that compounds such as arginine, glutamic acid, serine, glycine, alanine, and proline did not present significant changes ($p > 0.05$) during gastrointestinal digestion (GDI), which could be related to their resistance to hydrolysis or their early release in the digestive process. In contrast, tyrosine and phenylalanine increased their concentration after GDI, suggesting a selective release promoted by the action of pancreatic enzymes. Tryptophan, although present in low concentrations in the ND and GD samples, showed a modest increase in GID, probably due to the specific action of pancreatin on protein fragments containing this amino acid. According to Gong et al. (2022), pepsin plays a key role in the initial cleavage of large proteins, which subsequently facilitates pancreatin to degrade these fractions into small peptides and amino acids.

In the ND sample, essential amino acids accounted for 56.6% of the total amino acids, a percentage that increased to 80.9% after gastrointestinal digestion. Is higher than the essential amino acid content of goat whey studied by Borba et al. (2022) which represented about 44 % of the total content of BCAAs.

The present study confirmed an increase in BCAAs concentration during gastrointestinal digestion, from 6% at the beginning of digestion to 15% of the total amino acids released at the end of the digestive process. This increase is particularly relevant, as BCAAs play a crucial role in muscle protein synthesis, acting both as signaling molecules and direct substrates for this anabolic process (Kaspy et al., 2023). Zhao et al. (2024) established that the beneficial effect of hydrolyzed whey proteins on the regulation of muscle metabolism is associated with their low molecular weight fractions enriched in branched-chain amino acid

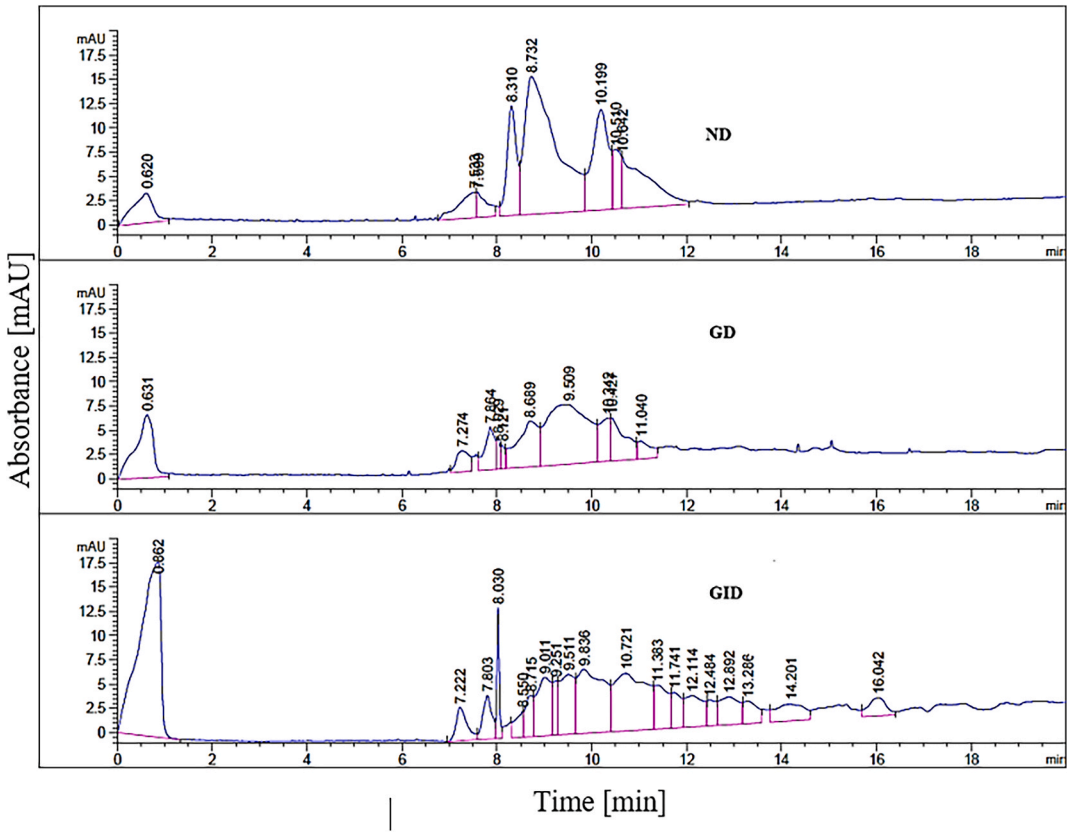

Fig. 3. Electrophoretic profile of goat whey protein concentrate at a concentration of 2.5 g/L. ND = Undigested, GD = gastric digest, and GID = gastrointestinal digest.

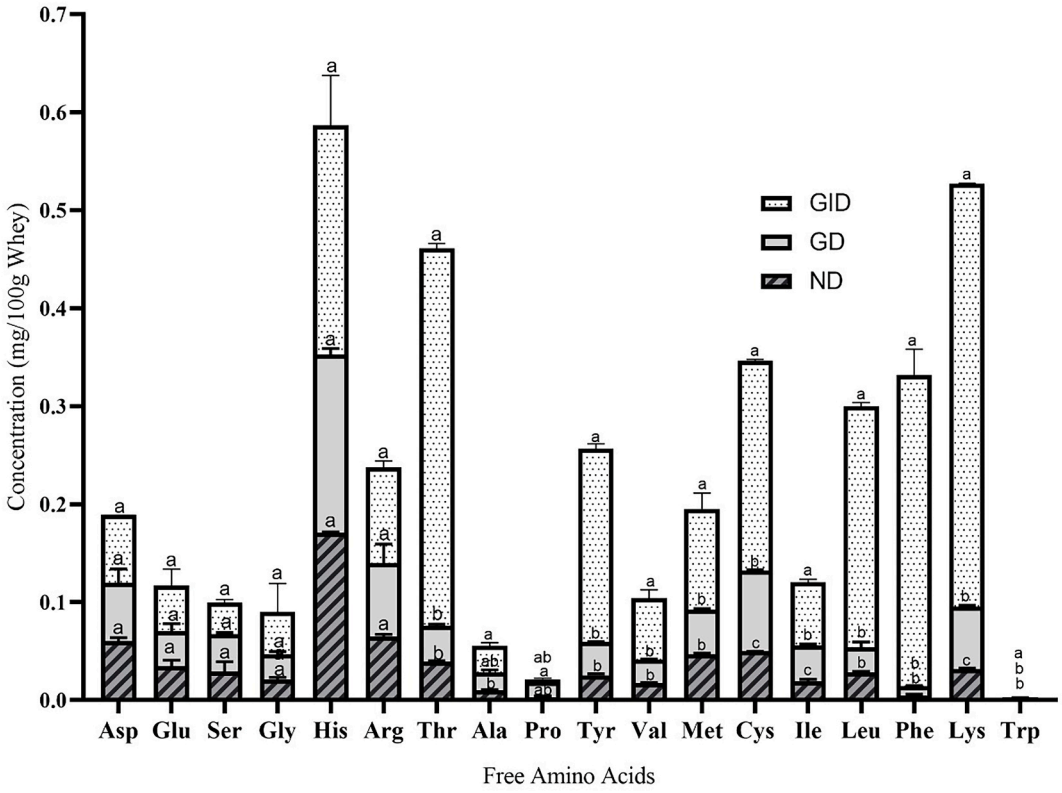

Fig. 4. Free amino acid profile (g/100g of sample) in the Undigested (ND), Gastric Digested (GD) and Gastrointestinal Digested samples (GID).

peptides. Furthermore, the rapid release and absorption of these peptides after whey digestion favors an efficient supply of essential nutrients to muscle tissue, optimizing its recovery and growth by improving carbohydrate and vitamin metabolism.

On the other hand, an increase in sulfur amino acids, such as methionine and cysteine, was observed during *in vitro* digestion. These amino acids are closely related to the Protein Efficiency Quotient (PEQ), an indicator of protein quality that reinforces the nutritional importance of digested whey. Sulfur amino acids, especially cysteine, present in whey proteins such as β-lactoglobulin, play a crucial role in the synthesis and regulation of glutathione. This tripeptide is essential for cellular defense against oxidative stress, mediating immune and hepatic functions, and possibly acting as a negative regulator in processes related to Alzheimer's disease and cancer (Campos et al., 2022). This aspect highlights the functional potential of digested whey for clinical and sports nutrition applications by providing bioactive compounds that optimize protein synthesis and cellular protection.

3.3. Antioxidant activity

The study of the antioxidant capacity of the protein concentrates, evaluated by $ABTS^{-+}$ and $DPPH^{-+}$ radicals, showed that the consumption of these radicals increased with the progress of digestion, indicating that the release of bioactive peptides increases the antioxidant activity (Table 2). The highest inhibition percentages were observed in GID, reaching 64.68% for $ABTS^{-+}$ and 47.95% for $DPPH^{-+}$. This behavior was similar to that observed by Cui et al. (2024), where the $ABTS^{-+}$ radical scavenging activity of the hydrolysates was significantly higher than the $DPPH^{-+}$ radical scavenging activity. This difference could be explained by the positive charge of the $ABTS^{-+}$ radical, which facilitates interaction with antioxidant substances.

The free radical scavenging capacity observed in goat whey hydrolysates by the $ABTS^{-+}$ method indicates that peptides generated during the gastric digestion (GD) and gastrointestinal digestion (GID) phases, under the action of pepsin and pancreatin enzymes, act as primary free radical scavengers in aqueous media. This phenomenon can be attributed to the hydrogen-donating capacity of peptides, which react with free radicals to form more stable products (Rodrigues et al., 2024). On the other hand, the antioxidant activity assessed by the $DPPH^{-+}$ assay is associated with the release of antioxidant compounds during GID, particularly aromatic amino acid residues such as tyrosine, phenylalanine, and tryptophan. These amino acids possess radical scavenging properties as they can donate protons to electron-deficient free radicals, stabilizing them and reducing their activity (Sakkas et al., 2022). The increase of these residues is shown in Fig. 3 as the *in vitro* digestion progressed.

The antioxidant activity observed (Fig. 2 and Table 2) of peptides generated by protein hydrolysis is likely due to the release of bioactive peptides with specific amino acid sequences, particularly those rich in aromatic (tyrosine, tryptophan, phenylalanine) or sulfur-containing (cysteine, methionine) residues. These amino acids can donate protons or neutralize free radicals, a fundamental mechanism in antioxidant activity (Sakkas et al., 2022). Previous studies have indicated that factors such as amino acid composition, structure, sequence, and physicochemical properties, including aromaticity and hydrophobicity, determine this functionality (Rakesh et al., 2019). In addition, smaller peptides generated during digestion have been reported to interact with bacterial membranes, disrupting their integrity due to their amphiphilic and cationic properties (Campos et al., 2022). In particular, amino acid composition plays a key role in the antioxidant capacity of peptides. Zhang et al. (2024), when using papain and alcalase in fresh defatted goat milk, observed that papain showed higher antioxidant activity, which underlines the influence of the type of enzyme used. In this study, the high antioxidant activity observed during gastrointestinal digestion (GID) can be attributed to the specificity of proteases present in pancreatin, capable of releasing bioactive peptides with high antioxidant potential (Espejo-Carpio et al., 2016). These results highlight the relevance of enzymatic processes to generating compounds with outstanding functional properties.

Table 2
Antioxidant activity of undigested and gastric and gastrointestinal digested goat whey protein concentrate.

Goat's milk whey	$DPPH^{\bullet+}$		$ABTS^{\bullet+}$	
	% Inhibition	Equivalent Trolox mg/g	% Inhibition	Equivalent Trolox mg/g
ND	44.48 ± 0.02^{b}	0.69 ± 0.03^{b}	36.62 ± 0.02^{c}	0.71 ± 0.03^{c}
GD	46.14 ± 0.03^{ab}	0.74 ± 0.02^{b}	46.26 ± 0.05^{b}	0.93 ± 0.01^{b}
GID	47.95 ± 0.01^{a}	2.00 ± 0.01^{a}	64.68 ± 0.03^{a}	2.70 ± 0.05^{a}

ND = Undigested; GD = Gastric Digested; GID = Gastrointestinal Digested.
[a,b,c] Different letters on the same line indicate a significant difference (Tukey test, $p < 0.05$).

Electrophoretic analysis (SDS-PAGE) provides indirect evidence of protein hydrolysis, as larger proteins are degraded during digestion. However, the method does not confirm the presence of smaller peptides, as they may fall below the detection limit of the gel (3.5 kDa) or may have been lost during sample preparation and analysis. Complementary techniques, such as reversed-phase HPLC and capillary electrophoresis, provide more robust evidence for the generation of peptides, as shown in Figs. 2 and 3. Future studies should incorporate mass spectrometry (e.g., LC-MS/MS) or other high-sensitivity methods to conclusively identify and quantify the smaller peptides generated during digestion. It would also be essential to include measurements of the degree of hydrolysis (DH) using established methods, such as OPA or TNBS assays to provide a quantitative assessment of protein digestion. These results will further elucidate the relationship between the extent of hydrolysis and the bioactive properties of the hydrolysates.

3.4. Antimicrobial activity

The antimicrobial activity of goat whey and its digests (GD and GID) was evaluated against various bacteria and yeasts, focusing on Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MBF) (Table 3). Digestion enhanced antimicrobial activity, as reflected by lower MIC and MBC values for GD and GID compared to undigested whey (ND). This effect is attributed to milk-derived bioactive peptides released during digestion by pepsin and pancreatin, which exhibit inhibitory effects on both Gram-positive and Gram-negative bacteria (Shivanna & Nataraj, 2020).

Regarding bacteriostatic activity, GD demonstrated bacteriostatic effects against *Escherichia coli* and *Listeria innocua* at 5 mg/mL and 2.5 mg/mL, respectively. GID inhibited *E. coli*, *Listeria innocua*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* at 0.625 mg/mL, 2.5 mg/mL, 5.0 mg/mL, and 5.0 mg/mL, respectively. As for bactericidal activity, GD showed bactericidal effects against *E. coli and Listeria innocua* at 10 mg/mL. GID exhibited bactericidal activity against *E. coli* and *P. aeruginosa* at the same concentration.

The enhanced activity, particularly against *E. coli*, aligns with findings by Lestari et al. (2022) on the antimicrobial potential of goat casein. This activity is linked to electrostatic interactions between positively charged peptides and negatively charged bacterial membranes, causing membrane disruption (Xiao et al., 2023). Factors influencing peptide antimicrobial efficacy include sequence, size, structure, isoelectric point, and enzymatic specificity (Rodrigues et al., 2024). The broader spectrum of activity observed after gastrointestinal digestion suggests that enzymatic hydrolysis releases peptides with immunomodulatory and antibacterial properties. Lactoferrin-derived peptides, for example, inhibit bacterial growth by depriving bacteria of iron, essential for their proliferation Campos et al., 2022; Xiao et al., 2023).

However, digested goat whey exhibited no antimicrobial activity against *Salmonella choleraesuis*, *Bacillus subtilis*, and *Candida albicans* at

Table 3
Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) in mg/mL of samples.

Samples	*Escherichia coli* ATCC 11775		*Salmonella choleraesuis* ATCC 10708 and *Bacillus subtilis* ATCC 5061		*Pseudomonas aeruginosa* ATCC 13388		*Staphylococcus aureus* ATCC 6538		*Listeria innocua* ATCC 33090		*Candida albicans* ATCC 10231	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ND	>10.0*	>10.0	>10.0	>10.0	10.0	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0
GD	5.0 ± 0.04	10.0 ± 0.02	>10.0	>10.0	>10.0	>10.0	>10.0	>10.0	2.5 ± 0.08	10.0 ± 0.04	>10.0	>10.0
GID	0.625 ± 0.01	10.0 ± 0.03	>10.0	>10.0	5.0 ± 0.01	10.0 ± 0.01	5.0 ± 0.05	>10.0	2.5 ± 0.10	>10.0	>10.0	>10.0

ND = Undigested; GD = Gastric Digested; GID = Gastrointestinal Digested; The concentration range of the hydrolysates tested was 10 to 0.001 mg/mL; when the highest concentration tested did not showed antimicrobial activity the results were expressed as > 10.0.

concentrations above 10 mg/mL, highlighting its limited effect on certain pathogens.

4. Conclusion

Goat whey protein concentrate was confirmed as a suitable material for obtaining protein hydrolysates by *in vitro* gastrointestinal simulation. The results revealed that the increase in the amount of peptides is directly related to the degree of digestion, showing higher activity in the intestinal phase (GID), attributed to the action of pancreatin, in contrast to the gastric phase (GD), dominated by pepsin. Essential and BCAAs were significantly released during digestion, underlining their nutritional importance. Furthermore, excellent antibacterial activity was observed, especially against *E. coli*, with significant antioxidant activity inhibiting $DPPH^{-+}$ and $ABTS^{-+}$ radicals. The undigested (ND) protein concentrate did not detect these bioactive properties. This study highlights the bioactive potential of peptides derived from goat whey protein concentrate under simulated *in vitro* digestion conditions, offering promising prospects for developing functional dairy protein-based products. Future research should focus on identifying the components responsible for the observed bioactive activities and understanding the mechanisms underlying these effects.

CRediT authorship contribution statement

Karla Kaligia Silva Borba: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carlos Gadelha:** Writing – original draft, Visualization, Validation, Project administration, Funding acquisition. **Katherine Gutiérrez-Álzate:** Writing – review & editing, Writing – original draft, Visualization, Validation. **Luciano da Silva Pinto:** Methodology, Formal analysis, Data curation. **Marta Sueli Madruga:** Writing – original draft, Validation, Software, Project administration, Methodology, Investigation. **Maria Teresa Bertoldo Pacheco:** Methodology, Investigation, Funding acquisition, Formal analysis. **Renata Maria Teixeira Duarte:** Formal analysis, Methodology, Project administration, Supervision, Writing – review & editing. **Rita de Cássia Ramos do Egypto Queiroga:** Writing – original draft, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Marion Pereira da Costa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis. **Marciane Magnani:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Tatiane Santi Gadelha:** Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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