International Dairy Journal 155 (2024) 105972

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Effect of *Lactiplantibacillus plantarum* CNPC003 and milk pasteurization on artisanal goat coalho cheese characteristics

Camila Neves Meireles Costa [a], Julia Mariano Caju de Oliveira [a], Angela Matilde da Silva Alves [b], Kaíque Yago Gervazio de Lima [c], Maria Isabel Ferreira Campos [d], Antônio Silvio do Egito [e], Karina Maria Olbrich dos Santos [f], Mônica Correia Gonçalves [g], Evandro Leite de Souza [h], Maria Teresa Bertoldo Pacheco [i], Adriane Elisabete Costa Antunes [j], Maria Elieidy Gomes de Oliveira [k, *]

[a] *Post-Graduate Program in Nutrition Sciences, Department of Nutrition, Federal University of Paraíba, 58051-900, João Pessoa, PB, Brazil*
[b] *Department of Food and Nutrition, Faculty of Food Engineering, University of Campinas, 13083-862, Campinas, SP, Brazil*
[c] *Post-Graduate Program in Natural Products and Bioactive Synthetics, Center of Health Sciences, Federal University of Paraíba, 58051-900, João Pessoa, PB, Brazil*
[d] *Department of Molecular Biology, Center for Exact and Natural Sciences, Federal University of Paraíba, 58051-900, João Pessoa, PB, Brazil*
[e] *Embrapa Goats and Sheep, Northeast Regional Center, 58428-095, Campina Grande, Brazil*
[f] *Brazilian Agricultural Research Corporation (Embrapa), 23020-470, Rio de Janeiro, RJ, Brazil*
[g] *Agrifood Science and Technology Center, Federal University of Campina Grande, 58840-000, Pombal, PB, Brazil*
[h] *Laboratory of Food Microbiology, Department of Nutrition, Federal University of Paraíba, 58051-900, João Pessoa, PB, Brazil*
[i] *Center of Science and Food Quality, Institute of Food Technology, 13070-178, Campinas, SP, Brazil*
[j] *School of Applied Sciences, State University of Campinas (FCA/UNICAMP), 13484-350, Limeira, SP, Brazil*
[k] *Laboratory of Bromatology, Department of Nutrition, Center of Health Sciences, Federal University of Paraíba, 58051-900, João Pessoa, PB, Brazil*

ARTICLE INFO

Article history:
Received 29 December 2023
Received in revised form
24 April 2024
Accepted 25 April 2024
Available online 30 April 2024

ABSTRACT

This study evaluated the effect of adding *Lactiplantibacillus plantarum* CNPC003 (LP) and applying milk pasteurization on the microbiological, technological, and physicochemical characteristics and protein profile of artisanal goat coalho cheese during 60 days of ripening. Four types of cheese were produced, including cheeses made with raw and pasteurized goat milk and with and without LP (RC, PC, RCLP, and PCLP). All cheeses were safe for consumption and maintained high lactic acid bacteria counts (above 8 log CFU mL^{-1}) at the end of the ripening period. RC and RCLP showed higher hardness and lower chewiness than cheeses made with pasteurized milk (PC and PCLP) after 60 days of ripening. As expected, there was a reduction in lactose content with a simultaneous increase in acidity and syneresis for all cheeses over the 60 days of ripening, with no significant change in the other evaluated physicochemical parameters. Proteolysis rates increased in all cheeses up to 60 days of ripening, aligning with elevated levels of soluble protein and free amino acids. After 60 days of ripening, PC showed a higher extent of proteolysis index, higher amount of high molecular weight peptides, higher number of identified proteins, and lower content of free amino acids. RCLP showed a higher depth of proteolysis index, higher free amino acids amount (1583 ± 3.95 mg 100 g^{-1}), and fewer identified proteins. Among the peptides identified, it was possible to define 30 potential bioactive peptides according to the bioactivity score (>0.5), where both cheeses added with LP had 18 of these peptides, mostly potentially ACE inhibition sequences. The addition of LP and the pasteurization of milk positively affected the microbiological safety of ripened coalho goat cheeses, primarily contributing to the differentiation of their texture and protein and bioactivity profile.

* Corresponding author.
E-mail address: mego@academico.ufpb.br (M.E.G. Oliveira).

https://doi.org/10.1016/j.idairyj.2024.105972
0958-6946/

1. Introduction

Goat milk has been part of the human diet for millennia, and in recent decades, its relevance has increased worldwide. This is due mainly to goat milk nutritional value and the health benefits it provides, such as better digestibility and lower allergenic properties when compared to cow milk, intestinal anti-inflammatory activity, and high contents of bioactive constituents, particularly peptides, conjugated linoleic acid, and oligosaccharides (Verruck, Dantas, & Prudencio, 2019).

Great emphasis has been placed on milk proteins as the main source of biologically active peptides. Milk protein hydrolysis through fermentation and ripening processes, and even during gastrointestinal digestion, can result in the release of peptides with several bioactivities, such as antimicrobial, antioxidant, immunomodulatory, opioid, mineral carrier, and antihypertensive activities (Balthazar et al., 2017; Coelho, Malcata, & Silva, 2022).

Cheese production is a good alternative to increasing goat milk consumption. Coalho cheese is a traditional Brazilian cheese characterized as semi-hard, white in color, with a typical open texture and salty and slightly acidic flavor. It can be made with raw or pasteurized cow or goat milk (Garcia, Oliveira, Queiroga, Machado, & Souza, 2012). Previous studies have reported the functional potential of goat coalho cheese linked to the presence of bioactive peptides (Fontenele, Bastos, Dos Santos, Bemquerer, & do Egito, 2017).

Coagulation of coalho cheese is obtained using rennet or proper coagulation enzymes, which can be complemented with lactic acid bacteria for technological purposes (Garcia et al., 2012). Coalho cheese is also a promising food matrix for probiotic delivery due to its fat and protein contents, which exert protective effects on added probiotics during gastrointestinal tract passage, contributing to the survival of these microorganisms until reaching the intestine, as the main probiotic target (Moraes, dos Santos, de Barcelos, Lopes, & do Egito, 2018). Therefore, cheeses added with probiotic lactic acid bacteria (LAB) could be food products with promising functional properties (Galdino et al., 2021).

Incorporation of LAB with probiotic and technological aptitudes in cheeses made with raw milk can influence the intrinsic microbial ecosystem and promote changes in texture, nutritional profile (protein and amino acidic profile), production of bioactive compounds, especially bioactive peptides, and improve microbiological safety and storability in these products (dos Santos et al., 2015; Jia et al., 2021).

The strain *Lactiplantibacillus plantarum* CNPC003, isolated from goat milk, has shown promising results regarding its resistance to simulated gastrointestinal tract conditions, antioxidant potential, bile salt deconjugation ability, good exopolysaccharide production, and technological aptitudes for dairy production, besides having genes associated with the ability to adhere to the intestinal mucosa and beta-galactosidase activity. Additionally, this strain could modulate the autochthonous microbiota of cheese made from raw milk and, consequently, increase its microbiological stability and modify and/or improve its nutritional characteristics, especially the protein/peptide profile (Barcelos, do Egito, dos Santos, de Moraes, & Teixeira Sá, 2020; Bomfim et al., 2020), representing a strain with probiotic potential to be demonstrated in subsequent studies.

Slow pasteurization of milk (65 °C for 30 min) for cheese production is a common practice in the dairy industry. The type of heat treatment of milk, adjunct dairy culture addition, and ripening time are influential factors affecting cheeses protein and peptide profile (Galli et al., 2019). Milk pasteurization eliminates pathogenic and spoilage microorganisms, but it is also part of the beneficial endogenous microbiota. In addition, milk pasteurization causes denaturation of whey proteins and inactivation/activation of native milk enzymes, causing changes in proteolysis and texture of cheeses made with pasteurized milk (Natrella, Gambacorta, Squeo, & Faccia, 2023).

This study has hypothesized that adding *L. plantarum* CNPC003 and using pasteurized goat milk could affect the microbiological, technological, physicochemical, and potential functional characteristics of artisanal goat coalho cheese, resulting in a potentially probiotic cheese with bioactive properties. To test this hypothesis, different formulations of goat coalho cheese were produced and evaluated regarding the effects of *L. plantarum* CNPC003 addition and milk pasteurization on their microbiological, technological, physicochemical, and protein profile during 60 days of ripening at 10 ± 1 °C.

2. Material and methods

2.1. Materials

Milk of native goats ("Parda Sertaneja" crossbreed) was obtained from Fazenda Carnaúba (Taperoá, PB, Brazil). Freeze-dried culture of *L. plantarum* CNPC003 (formerly *Lactobacillus plantarum* B12, Genetic Heritage: BRMCTAA179), previously isolated from goat milk (Anglo-Nubian breed), belonging to the "Collection of Microorganisms of Interest to the Food Industry", was provided by Embrapa Goat and Sheep (Sobral, CE, Brazil). Liquid rennet (Ha-La, microbial enzyme chymosin – *Aspergillus niger* var *awamori*, 1:3000/75 IMCU), lactic acid (Christian Hansen®, Valinhos, MG, Brazil), calcium chloride P.A. (FMaia® Ltda, Cotia, SP, Brazil), and refined iodized salt (Norte Salineira S. A. Ind. and Com., Areia Branca, RN, Brazil) were purchased commercially.

To ensure cheese processing quality, the goat milk was characterized regarding physicochemical (Table S1) and microbiological (Table S2) parameters. Both raw and pasteurized goat milk met the physicochemical and microbiological standards set by Brazilian legislation (Brazil, 2000).

2.2. L. plantarum *CNPC003 inoculum preparation and goat coalho cheese processing*

Before starting cheese processing, the inoculum was prepared and later added to the cheese preparation. *L. plantarum* CNPC003 strain was aseptically added at a concentration of 0.2% (w/v) in powdered whole goat milk (Caprilat®, Governador Valadares, MG, Brazil) diluted in sterilized water (0.13%, w/v), cooled to 35 ± 0.5 °C, and incubated at 37 °C for 24 h. To enumerate the viable cell count of *L. plantarum* CNPC003, an aliquot of the starter preparation was taken, serially diluted (1:9, 10^{-1} to 10^{-9}) in sterilized peptone water (HiMedia, Mumbai, India) at 0.1% (w/v), inoculated in de Man, Rogosa, and Sharpe agar (MRS, HiMedia), and incubated at 37 °C for 48 h. The final viable cell count of *L. plantarum* CNPC003 in the inoculum to be added to the milk bulk for cheese processing was >8 log CFU mL^{-1}.

Four types of goat coalho cheese (treatments) were produced, namely: RC – raw goat milk cheese without *L. plantarum* CNPC003; RCLP – raw goat milk cheese with *L. plantarum* CNPC003; PC – pasteurized goat milk cheese without *L. plantarum* CNPC003; and PCLP – pasteurized goat milk cheese with *L. plantarum* CNPC003. Goat coalho cheeses were prepared according to a previously described procedure (Laguna & Landim, 2003). Firstly, goat milk was mixed, and a half part was kept raw (RC and RCLP cheeses processing), while the other half part was submitted to slow pasteurization (65 °C/30 min – PC and PCLP processing). For each independent processing, a total of 48 L of goat milk was used. Both raw and pasteurized milk were brought to 37 °C to start cheese processing. Then, lactic acid (85%) was added [0.25 mL L^{-1},

previously diluted in the proportion of 1:10 (v/v)], the activated inoculum of *L. plantarum* CNPC003 was added (20 mL L^{-1} of milk) for RCLP and PCLP cheeses, mixed, and calcium chloride solution (50%, 0.5 mL L^{-1}) and liquid rennet (0.9 mL L^{-1}) were added. The milk was mixed and passed through coagulation (40 min), cutting, mixing, salting, and draining. The mass was placed in perforated rectangular molds, pressed for 4 h, underwent a drying process (4 ± 0.5 °C for 1 h) in a B.O.D. (Marconi, MA 415, Piracicaba, SP, Brazil), and vacuum packaged (Tecmaq, TM 150, São Paulo, SP, Brazil).

Three independent processes were carried out for each type of cheese manufactured, which were analyzed in triplicate at 1, 20, 40, and 60 days of ripening at 10 ± 1 °C regarding the microbiological, technological, physicochemical, and chemical characteristics (protein, peptide, and amino acidic profile). Each cheese block produced weighed around 400 g and measured 12 × 7 × 6 cm. For each analysis, samples were taken from both the cheese core and rind.

2.3. Determination of goat coalho cheese characteristics

2.3.1. Microbiological parameters

2.3.1.1. Evaluation of hygienic-sanitary quality and viable cell counts of lactic acid bacteria during ripening. The analysis of thermotolerant coliforms, coagulase-positive staphylococci, and detection of *Salmonella* sp. per 25 g^{-1} and *Listeria monocytogenes* per 25 g^{-1} were performed using standard procedure (Salfinger & Tortorello, 2015). Samples were analyzed on the first day after processing and at 20, 40, and 60 days of ripening since the current Brazilian legislation allows the commercialization of cheeses made with raw milk only after 60 days of ripening (Brazil, 1996). A detection limit of <1 log CFU g^{-1} was employed for coagulase-positive staphylococci count and <3 MPN g^{-1} for the most probable number (MPN) of thermotolerant coliforms.

The LAB viable cell count was enumerated at 1, 20, 40, and 60 days of cheese ripening under 10 ± 1 °C using previously described procedures (Moraes et al., 2018; Salfinger & Tortorello, 2015). Initially, 25 g of cheese samples were collected aseptically from different parts of the cheeses, added and homogenized in 225 mL of sterilized peptone water (1 g L^{-1}, HiMedia), 1 mL aliquots of each mixture were serially diluted (1:9, 10^{-1} to 10^{-9}) in sterilized peptone water (1 g L^{-1}, HiMedia), 1 mL aliquots of each dilution were plated on MRS agar (HiMedia) supplemented with cysteine (0.5% w/v, Sigma–Aldrich Co., St. Louis, USA) (for cheeses with *L. plantarum* CNPC003), and incubated at 37 °C for 48 h under anaerobiosis (Anaerogen System Anaerogen, Oxoid). LAB viable cell counts were expressed as a log of the colony-forming units per gram of cheese (log CFU g^{-1}).

2.3.2. Determination of technological and physicochemical parameters

2.3.2.1. Yield and syneresis evaluation. After processing, the yield and syneresis of each batch of goat coalho cheese were calculated based on the equations presented below. The yield of coalho cheese was expressed (in g of cheese L^{-1} of milk) as the fresh cheese weight in grams obtained from 1 L of milk. Syneresis (in g 100 g^{-1}) at different ripening times (1, 20, 40, and 60 days of ripening) was calculated as the weight of whey in grams released by each gram of cheese in its own packaging divided by the cheese weight in grams in the same packaging and multiplied by 100 (Oliveira, Garcia, Queiroga, & Souza, 2012).

$$\text{Yield} = \frac{\text{Weight in grams of cheese}}{\text{Liters of milk}} \quad (1)$$

$$\text{Syneresis} = \frac{\text{Weight in grams of whey} \times 100}{\text{Weight in grams of cheese}} \quad (2)$$

2.3.2.2. Instrumental texture evaluation. The instrumental texture analysis sampling was conducted in triplicate on the entire pieces of each examined goat coalho cheese formulation cut at different positions using a cylindrical stainless-steel mold with dimensions of 2 cm in height and 5 cm in diameter. Cheese instrumental texture was determined through a two-bite compression test with a CT3 texturometer (Brookfield Engineering Labs, Middleboro, USA) using a cylindrical acrylic device with 25 mm in diameter and performing compression of 1 cm. The speed used was 1 mm s^{-1}. Data were collected from the Texture Expert for Windows program – version 1.20 (Stable Micro Systems). Hardness, cohesiveness, adhesiveness, elasticity, chewiness, and gumminess were measured. The following parameters were used: goat coalho cheese samples with height and diameter of 2 cm and 5 cm, respectively; temperature of 10 ± 1 °C; and distance and compression speed of 10 mm and 1 mm s^{-1}, respectively (Oliveira et al., 2012).

2.3.2.3. Determination of physical and physicochemical parameters. pH, titratable acidity in lactic acid, moisture, total dry extract, ash, protein, lactose, and sodium chloride contents were measured with standard procedures (AOAC, 2019), and fat content was measured using the Folch, Lees, and Sloane Stanley (1957) method.

2.3.3. Analysis of protein, amino acidic, and peptide profile

Initially, samples (200–300 g) were lyophilized in a benchtop freeze dryer (L-101 model; LIOTOP®, São Carlos, SP, Brazil) at a temperature of −55 ± 2 °C, vacuum pressure <138 μHG, lyophilization speed of 1 mm h^{-1}, and drying time of approximately 48 h. Freeze-dried samples were evaluated according to the methods described below.

2.3.3.1. Soluble protein. Total soluble protein content in cheese at 1, 20, 40, and 60 days of ripening was determined as previously described (Bradford, 1976). Freeze-dried cheese samples were diluted in different concentrations of NaCl at 0.15 mol L^{-1} (1:10, 1:20, 1:40, 1:80, 1:160, 1:360). An aliquot of 100 μL from each dilution was added of 2.5 mL of Bradford reagent, homogenized, left to rest for 10 min, and read at 595 nm in a spectrophotometer (Varian Cary® 50 UV–Vis Spectrophotometer, Australia). A curve of BSA–bovine serum albumin was used as standard.

2.3.3.2. Proteomic analysis

2.3.3.2.1. Preparation of cheese sample. Approximately 100 mg of each freeze-dried cheese sample obtained at 60 days of ripening were weighed and diluted in 1 mL of 150 mM Tris–HCl buffer, pH 8.8, containing 8 M urea and O.G. (1-S-octyl-β-D-thioglucopyranoside) at 0.1%, resulting in samples at a concentration of 100 mg mL^{-1}. The samples were centrifuged (12,000× *g*, 4 °C, 5 min, Centrifuge model RC5C, Sorvall Instruments Dupont, Wilmington, USA), and 100 μL of the supernatant was subjected to protein precipitation using acetone. 400 μL of acetone:ethanol:formic acid solution (50:49.5:0.5, respectively) were added, and the sample was kept overnight in a freezer. After centrifugation (12,000× *g*, 4 °C, 10 min), the pellet containing the proteins was resuspended in 100 μL of Tris–HCl buffer at 150 mM, pH 8,8, containing 8 M urea and 0.1% O.G.

2.3.3.2.2. SDS-PAGE electrophoresis – general evaluation of the protein profile and casein protein separation. Polyacrylamide gel

electrophoresis (SDS-PAGE) was used for general evaluation of the protein profile in freeze-dried cheese samples, as well as for separation of the band corresponding to casein protein. Approximately 50 μg of each sample was resuspended in XT 1× electrophoresis buffer (Bio-Rad, cod. 161-0791) containing 5% β-mercaptoethanol (v/v, Sigma) for the reduction of protein disulfide bridges. After incubation at 100 °C for 5 min, samples were loaded onto 8–16% polyacrylamide gels (Genescript). Electrophoresis was performed with 40 V/gel for 30 min and 150 V for 1 h in a Mini-PROTEAN II Electrophoresis System (Bio-Rad, USA). 5 μL of a duo color molecular weight standard (Bioread) was loaded. Gels were stained with Coomassie Gel Code Blue Stain Reagent (Thermo, code 24590) according to manufacturer recommendations to visualize the proteins.

2.3.3.2.3. Tryptic digestion of cheese samples from SDS gel. Cheese samples were submitted to in-gel tryptic digestion for analysis by advanced mass spectrometry. Casein corresponding band was clipped and excluded from the analysis. Initially, bands were extensively washed with a 50 mM ammonium bicarbonate solution containing 50% (v/v) acetonitrile to remove SDS and dye, subjected to protein disulfide bridges reduction by adding 50 μg of DTT (dithiothreitol) (GE), incubated for 1 h at 37 °C, submitted to alkylation by adding 250 μg of iodoacetamide (Sigma Ultra), and incubated for 1 h at room temperature (25 ± 2 °C) in the dark. Bands were dried in a speed vac and incubated with 0.2 μg of trypsin (Sigma) diluted in 50 mM ammonium bicarbonate. Digestion was performed at 37 °C for approximately 18 h. Extraction of the resulting peptides was initially performed twice with a solution containing 50% acetonitrile (v/v) and 2% formic acid (v/v), followed by re-extraction with a solution containing 100% acetonitrile and 2% acid formic. Before the sample was applied in the mass spectrometer, clean-up/desalting was performed using an OASIS HLB Cartridge 1 cc column (cat. number: 186000383, Waters) as described by the manufacturer. Briefly, the column was equilibrated with a 5% acetonitrile solution containing 0.1% formic acid, and the material was eluted with 80% acetonitrile.

2.3.3.2.4. Peptides identification by advanced mass spectrometry. Digested samples were analyzed by an Orbitrap Eclipse mass spectrometer (ThermoFisher) coupled to a Nano LC–MS/MS nanoflow chromatography system (Dionex Ultimate 3000 RLSCnano System, Thermofisher). Approximately 1 μg of the sample was injected for analysis. Peptides were separated on a Nanoease MZ peptide BEH C18 column (130A, 1.7 μm, 75 μm × 250 mm, Waters), at a continuous flow rate of 300 nL min^{-1}, using a gradient of 4–50% acetonitrile in 90 min. Data were acquired on MS1 in the range of M/Z 375–1500 (120,000 resolution, AGC target 1 E6 with maximum injection time of 100 ms). The most abundant ions were submitted to MSMS with collision energy of 30%, precursion isolation window of 1.2 m/z, AGC target 1E5, and resolution of 15,000. Raw data were converted to mzXML and processed using the Comet algorithm (v. 2018). Uniprot database was used. Identified peptides reaching the maximum criterion of 3% error by the Peptide Prophet were used to extract the intensity for quantification by the Xpress algorithm. Peptides and their intensities were grouped to generate protein intensity using a script in R language.

The peptides identified were evaluated by the Peptide Ranker server, which is based on a new N-to-1 neural network based on the probability that the peptide will be bioactive. This server has a calibration threshold of 0.5, meaning that any peptide predicted above this value is labeled bioactive (Mooney, Haslam, Pollastri, & Shields, 2012). Peptide bioactivity was also predicted using the BIOPEP database to estimate the potential for biological activity.

The physicochemical characteristics of the peptide sequences obtained were evaluated using the following tools: Expasy-Compute (http://web.expasy.org/compute_pi/) used to estimate the isoelectric point and the molecular mass (Da). The grand average hydropathicity (GRAVY), neutral charge, and hydrophobicity were estimated using the PepDraw tool (https://pepdraw.com/).

Based on the obtained results, the peptides with the greatest potential for bioactivity were selected by representing the primary sequence using the PepDraw tool and the 3D structure using the UCSF ChimeraX software: "Tools for structure building and analysis." (Meng EC, Goddard TD, Pettersen EF, Couch GS, Pearson ZJ, Morris JH, Ferrin TE. Protein Sci).

2.3.3.3. Total and free amino acids. Determination of total amino acids in cheese samples at 1 and 60 days of ripening was carried out by reverse phase column chromatography in a high-performance liquid chromatography system (HPLC, SHIMADZU brand, Prominence model), as previously described (Hagen, Frost, & Augustin, 1989; White, Hart, & Fry, 1986). To release individual amino acids, the freeze-dried sample was resuspended in 9 mL of 6 M hydrochloric acid and phenol. After hydrolysis (110 °C/22 h), α-aminobutyric acid (AABA) was added as an internal standard. The acid was eliminated by vacuum evaporation, and the hydrolyzate was derivatized with a methanol solution, ultrapure water, triethylamine, and phenylisothiocyanate (PITC). Amino acids were filtered through a 0.22 μm filtering membrane (Milipore, Billerica MA, USA) and introduced into the column. The mobile phase consisted of a mixture of eluents A (sodium acetate – pH 6.4) and B (50% acetonitrile, ultrapure water, disodium EDTA).

Individual amino acids separation took place according to the following chromatographic conditions: analytical column: LUNA C18 100 A 5 μM 250 × 4.6 mm 00G-4252-EQ, eluents flow (mobile phase): 1 mL min^{-1} at 50 °C, DAD detector (diode array), with detection at 254 nm. Amino acid elution order was as follows: Aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, internal standard (AABA), tyrosine, valine, methionine, cystine (3 peaks), isoleucine, leucine, phenylalanine, and lysine. Compound peak identification was performed by comparing retention times obtained in injected standards. Amino acids identification was carried out by comparison with an external standard (Pierce, PN 20088), and internal standard α-aminobutyric acid (Sigma–Aldrich) was used for quantification.

For free amino acids determination, samples were mixed with methanol (60%) and 0.1 M HCl (30%), and the mixture was subjected to orbital agitation for 30 min. After filtration, an aliquot of the filtrate was derivatized following the same method as for total amino acids (except acid hydrolysis with high temperature). Tryptophan determination was carried out separately, as this amino acid degrades in acid hydrolysis. For this purpose, enzymatic hydrolysis was performed with 0.4 mL of pronase solution and 3.6 mL of 0.1 M sodium phosphate buffer solution (40 °C/22–24 h), followed by a colorimetric reaction with 4-dimethylaminobenzaldehyde in 21.2 N sulfuric acid (H_2SO_4), kept in the dark at room temperature (25 ± 2 °C) for 6 h. Subsequently, 0.1 mL of $NaNO_2$ solution was added, and 30 min later, the absorbance was read at 590 nm in a spectrophotometer (Varian Cary® 50 UV–Vis Spectrophotometer, Australia). Tryptophan content was calculated from a standard curve of L-tryptophan (Spies, 1967).

2.3.3.4. Proteolysis determination. Proteolysis was evaluated by extent and depth indexes. The extent of the proteolysis index (EPI) is mainly related to milk natural proteinases and the coagulating agent (chymosin and renin, for example) used, which degrade the protein into high molecular weight peptides. The depth of proteolysis index (DPI) is mainly related to the activity of endoenzymes and exoenzymes from lactic acid culture used in cheese manufacturing, from milk natural microbiota (cheese made from

raw milk), and from possible contaminants, which degrade peptides of low molecular weight.

Indexes were obtained through calculations described below (Wolfschoon-Pombo, 1983):

$$\text{Extent Proteolysis Index}\ (\%) = \left(\frac{\text{NCN}}{\text{TN}}\right) \times 100 \quad (3)$$

$$\text{Depth of Proteolysis Index}\ (\%) = \left(\frac{\text{NPN}}{\text{TN}}\right) \times 100 \quad (4)$$

The Micro Kjeldahl method was used for TN (total nitrogen) determination (AOAC, 2019). NPN (non-protein nitrogen) determination was carried out by grinding 1 g of the fresh cheese sample, with the aid of a turrax (Potter Type Homogenizer, TE-102, Tecnal, Piracicaba, SP, Brazil) for 40–60 s in 50 mL of 12% trichloroacetic acid (TCA). After protein precipitation, filtration was performed on a 0.45 μm filter paper (Whatman®, GE 49 Healthcare, Chicago, IL, USA), and a 5 mL filtrate aliquot was added to a Kjeldahl tube, along with a catalyst, and forwarded to the digestion process. Tubes were removed from the digester, and after cooling to room temperature, distillation, and titration processes were carried out according to the micro Kjeldahl method (AOAC, 2019).

Determination of NCN (non-casein nitrogen) was also carried out by grinding 1 g of the fresh cheese sample for 40–60 s, with the aid of a turrax (Tecnal). However, for casein precipitation, 50 mL of working solution (pH 4.6), composed of distilled water, sodium acetate, sodium chloride, calcium chloride, and acetic acid, was used. Filtration was performed on a 0.45 μm filter paper (Whatman®), and the filtrate was subjected to the process of digestion, distillation, and titration to obtain soluble nitrogen (AOAC, 2019).

2.4. Statistical analysis

Experiments were performed in triplicate in two independent experiments, and results were expressed as average ± standard deviation. The Kolmogorov–Smirnov normality test was performed to verify the normal distribution of data. Data were submitted to Student's t-test or analysis of variance (ANOVA) followed by Tukey's test with $P < 0.05$. A principal component analysis (PCA) was performed to visualize the correlation among cheese samples and free amino acids, proteolysis index, total identified proteins, and potential bioactive peptides after ripening for 60 days at 10 °C. Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Microbiological parameters of goat coalho cheese

Table 1 shows the results of the measured microbiological parameters in examined goat coalho cheese during 60 days of ripening at 10 ± 1 °C. In general, cheeses made with pasteurized milk showed better microbiological quality from the beginning of ripening regarding the counts of coagulase-positive staphylococci (<1 log CFU g^{-1}) and MPN of thermotolerant coliforms (<3 MPN g^{-1}). These results demonstrate the effects of pasteurization on reducing the microbial load, especially pathogenic and spoilage microorganisms, reinforcing the efficacy of thermal treatment in controlling the hygienic-sanitary quality of the food matrix. Cheeses made with raw milk reduced the MPN of thermotolerant coliforms at 60 days of ripening, with greater reductions in cheeses with *L. plantarum* CNPC003 (RCLP), reaching <3 MPN g^{-1}. Coalho cheeses made with raw milk (RC and RCLP) had counts of coagulase-positive staphylococci ranging from 4.98 ± 0.41 to 5.11 ± 0.11 log CFU g^{-1} on the first day of ripening. However, these counts dropped to <1 log CFU g^{-1} at 60 days of ripening, with greater reductions at day 20 of ripening in cheese with *L. plantarum* CNPC003 (RCLP). These results show the efficacy of *L. plantarum* CNPC003 in enhancing the microbiological quality of cheeses made from raw milk, which was comparable to cheeses made from pasteurized milk. This result may be associated with the antimicrobial activity of *L. plantarum*, as previously observed in Iranian

Table 1
Microbiological parameters of goat coalho cheese made with raw or pasteurized milk and added or not with *Lactiplantibacillus plantarum* CNPC003 during 60 days of ripening (10 ± 1 °C).

Microbiological parameters	Ripening time (days)	Cheeses			
		RC	RCLP	PC	PCLP
Coagulase-positive staphylococci (log CFU g^{-1})	1	5.11 ± 0.11^{aA}	4.98 ± 0.41^{bA}	<1	<1
	20	3.70 ± 0.1^{B}	<1	<1	<1
	40	<1	<1	<1	<1
	60	<1	<1	<1	<1
Listeria monocytogenes (absence in 25 g)	1	Absent	Absent	Absent	Absent
	20	Absent	Absent	Absent	Absent
	40	Absent	Absent	Absent	Absent
	60	Absent	Absent	Absent	Absent
Salmonella spp. (absence in 25 g)	1	Absent	Absent	Absent	Absent
	20	Absent	Absent	Absent	Absent
	40	Absent	Absent	Absent	Absent
	60	Absent	Absent	Absent	Absent
Thermotolerant coliforms (MPN g^{-1})	1	290	210	<3.0	23
	20	38	21	<3.0	<3.0
	40	35	16	<3.0	<3.0
	60	3.6	<3.0	<3.0	<3.0
LAB count[a] (log CFU g^{-1})	1	8.13 ± 0.03^{aA}	8.32 ± 1.01^{aA}	6.38 ± 0.72^{bB}	6.09 ± 0.12^{bB}
	20	9.07 ± 0.07^{aA}	8.37 ± 0.09^{aA}	8.87 ± 0.14^{aA}	8.29 ± 0.09^{aA}
	40	9.04 ± 0.40^{aA}	8.36 ± 0.10^{aA}	8.89 ± 0.13^{aA}	8.78 ± 0.06^{aA}
	60	8.58 ± 0.10^{aA}	8.16 ± 0.01^{aA}	8.97 ± 0.04^{aA}	8.30 ± 0.13^{aA}

Cheeses: RC – raw milk cheese; RCLP – raw milk cheese added with *L. plantarum* CNPC003; PC – pasteurized milk cheese; PCLP – pasteurized milk cheese added with *L. plantarum* CNPC003.
a–b: average ± standard deviation with different lowercase letters within a row differ ($P < 0.05$) among cheese formulations, based on Tukey's test or Student's t-test.
A–B: average ± standard deviation with different uppercase letters within a column differ ($P < 0.05$) among days of ripening, based on Tukey's test or Student's t-test.
[a] Lactic acid bacteria total count.

Koozeh cheese made with raw goat or sheep milk (Jabbari et al., 2017).

The ability of some LAB strains to inhibit pathogenic bacteria through the production of organic acids, bacteriocins, carbon dioxide, ethanol, hydrogen peroxide, and diacetyl makes these microorganisms important tools in the development of potentially functionalized and safe foods. The microbial load reduction in cheeses at 60 days of ripening could be linked to the production of antimicrobial peptides by *L. plantarum* CNPC003, such as bacteriocins, that reduce dairy product microbial contamination (Coelho et al., 2022), as well as the increase in acidity during ripening (Table 2). *Salmonella* spp. and *L. monocytogenes* were not detected in coalho cheese samples.

The count of LAB, especially of probiotics, in dairy products is an important indicator of functional quality (Kisan et al., 2021; Terpou et al., 2019). The total LAB counts for all the examined coalho cheese samples were greater than 8 log CFU mL^{-1} at 60 days of ripening. Cheeses with *L. plantarum* CNPC003 (RCLP and PCLP) had counts above 6 log CFU g^{-1} already from day 1 onward, which is the minimum count commonly recommended in products with probiotics to provide health benefits to consumers (Terpou et al., 2019). A total LAB count was performed because the analysis conditions were not selective for *L. plantarum.* In addition to health benefits, probiotics add value to dairy products by improving their technological, sensory, nutritional, and microbiological characteristics (Gao et al., 2021).

Dairy products, such as cheeses, yogurts, and other fermented beverages, are among the foods with great potential to carry probiotic bacteria and maintain expected counts during storage (Terpou et al., 2019). Cheeses stand out among the products mentioned as excellent carriers of probiotics due to their slightly and naturally acidic pH, high moisture content, high fat content, and solid consistency, which provide mechanical protection for microorganisms during processing, ripening, storage, and passage through the gastrointestinal tract (Kisan et al., 2021). Indeed, the examined coalho cheese samples maintained high LAB counts until

Table 2
Technological and physicochemical characteristics of goat coalho cheese made with raw or pasteurized milk and added or not with *Lactiplantibacillus plantarum* CNPC003 during 60 days of ripening (10 ± 1 °C).

Parameters	Ripening time (days)	Cheeses			
		RC	RCLP	PC	PCLP
Yield (g L^{-1} of milk)	1	157 ± 15.7^{a}	160 ± 12.9^{a}	166 ± 8.7^{a}	162 ± 5.9^{a}
Syneresis (g of whey 100 g^{-1})	1	0.60 ± 0.25^{aB}	0.93 ± 0.55^{aB}	1.50 ± 0.39^{aB}	2.47 ± 0.40^{aB}
	20	2.42 ± 0.58^{bA}	5.20 ± 0.50^{aAB}	4.20 ± 0.50^{abA}	4.15 ± 0.55^{abAB}
	40	2.69 ± 0.26^{bA}	7.95 ± 0.45^{aA}	6.66 ± 0.55^{aA}	4.31 ± 0.54^{bAB}
	60	2.70 ± 0.50^{cA}	8.49 ± 0.50^{aA}	7.76 ± 0.55^{aA}	4.68 ± 0.32^{bA}
pH	1	6.60 ± 0.01^{abA}	6.40 ± 0.07^{abA}	6.78 ± 0.07^{aA}	6.21 ± 0.04^{bA}
	20	5.39 ± 0.02^{aB}	5.26 ± 0.06^{aAB}	5.69 ± 0.07^{aB}	5.53 ± 0.01^{aB}
	40	5.23 ± 0.01^{cC}	5.21 ± 0.07^{cB}	5.56 ± 0.02^{aB}	5.39 ± 0.01^{bC}
	60	5.08 ± 0.03^{bC}	5.05 ± 0.03^{bB}	5.42 ± 0.04^{aB}	5.25 ± 0.07^{abC}
Titratable acidity (g 100 g^{-1})	1	0.04 ± 0.01^{aB}	0.04 ± 0.01^{aB}	0.02 ± 0.01^{aA}	0.04 ± 0.01^{aA}
	20	0.15 ± 0.01^{aAB}	0.18 ± 0.01^{aA}	0.09 ± 0.01^{bA}	0.14 ± 0.01^{aA}
	40	0.16 ± 0.01^{aAB}	0.17 ± 0.01^{aA}	0.10 ± 0.01^{bA}	0.14 ± 0.01^{abA}
	60	0.17 ± 0.01^{aA}	0.18 ± 0.01^{aA}	0.10 ± 0.01^{bA}	0.15 ± 0.01^{abA}
Moisture (g 100 g^{-1})[b]	1	48.6 ± 0.49^{bA}	47.2 ± 0.58^{bA}	51.7 ± 0.52^{aA}	50.6 ± 0.93^{abA}
	20	49.9 ± 0.86^{abA}	45.4 ± 0.01^{cA}	51.8 ± 0.59^{aA}	47.1 ± 0.66^{bcA}
	40	47.2 ± 0.54^{cA}	48.3 ± 0.42^{bcA}	52.0 ± 0.34^{aA}	50.4 ± 0.32^{abA}
	60	49.1 ± 0.56^{bA}	44.8 ± 0.67^{cA}	52.8 ± 0.47^{aA}	50.6 ± 0.76^{abA}
TDE[a] (g 100 g^{-1})	1	51.4 ± 0.49^{aA}	52.8 ± 0.58^{aA}	48.3 ± 0.52^{bA}	49.5 ± 0.93^{abA}
	20	50.1 ± 0.86^{bcA}	54.6 ± 0.01^{aA}	48.2 ± 0.59^{cA}	52.9 ± 0.66^{abA}
	40	52.9 ± 0.54^{aA}	51.7 ± 0.42^{abA}	48.0 ± 0.34^{cA}	49.6 ± 0.32^{bcA}
	60	50.9 ± 0.56^{bA}	55.2 ± 0.67^{aA}	47.2 ± 0.47^{cA}	49.4 ± 0.76^{bcA}
Ashes (g 100 g^{-1})	1	2.91 ± 0.17^{aA}	2.71 ± 0.14^{aA}	2.47 ± 0.43^{aA}	3.63 ± 0.57^{aA}
	20	2.65 ± 0.52^{aA}	2.52 ± 0.04^{aA}	2.54 ± 0.09^{aA}	3.47 ± 0.56^{aA}
	40	2.68 ± 0.15^{aA}	2.98 ± 0.21^{aA}	2.60 ± 0.05^{aA}	3.31 ± 0.41^{aA}
	60	2.42 ± 0.11^{aA}	2.38 ± 0.04^{aA}	2.47 ± 0.13^{aA}	2.78 ± 0.17^{aA}
Protein (g 100 g^{-1})	1	21.6 ± 0.35^{aA}	21.3 ± 0.45^{aA}	17.7 ± 0.36^{bA}	20.3 ± 0.73^{aA}
	20	18.2 ± 0.35^{bC}	20.9 ± 0.55^{aA}	18.5 ± 0.79^{bA}	19.2 ± 0.32^{abA}
	40	19.1 ± 0.57^{aBC}	19.7 ± 0.23^{aA}	18.8 ± 0.39^{aA}	18.8 ± 0.72^{aA}
	60	19.1 ± 0.19^{aB}	19.8 ± 0.45^{aA}	19.0 ± 0.16^{aA}	19.8 ± 0.14^{aA}
Fat (g 100 g^{-1})[c]	1	22.4 ± 0.02^{bB}	23.8 ± 0.08^{aC}	18.9 ± 0.09^{dC}	19.6 ± 0.10^{cD}
	20	22.9 ± 0.04^{bB}	23.9 ± 0.04^{aC}	20.5 ± 0.07^{cBC}	21.5 ± 0.01^{cC}
	40	24.0 ± 0.06^{bA}	24.9 ± 0.01^{aB}	22.1 ± 0.13^{cAB}	21.9 ± 0.01^{cB}
	60	24.4 ± 0.04^{bA}	25.2 ± 0.01^{aA}	24.3 ± 0.02^{bA}	23.6 ± 0.06^{cA}
Lactose (g 100 g^{-1})	1	2.02 ± 0.16^{bc}	1.97 ± 0.02^{b}	2.84 ± 0.07^{aA}	1.46 ± 0.03^{cA}
	20	<LD	<LD	1.29 ± 0.02^{aB}	0.30 ± 0.01^{bB}
	40	<LD	<LD	1.01 ± 0.04^{B}	<LD
	60	<LD	<LD	0.75 ± 0.06^{B}	<LD
NaCl (g 100 g^{-1})	1	0.58 ± 0.26^{aA}	0.80 ± 0.18^{aA}	0.98 ± 0.06^{aA}	1.60 ± 0.71^{aA}
	20	0.54 ± 0.19^{aA}	0.54 ± 0.18^{aA}	0.81 ± 0.18^{aAB}	1.06 ± 0.67^{aA}
	40	0.40 ± 0.10^{aA}	0.33 ± 0.10^{aA}	0.31 ± 0.05^{aB}	1.08 ± 0.40^{aA}
	60	0.30 ± 0.04^{aA}	0.27 ± 0.14^{aA}	0.34 ± 0.09^{aB}	0.61 ± 0.07^{aA}

Cheeses: RC – raw milk cheese; RCLP – raw milk cheese added with *L. plantarum* CNPC003; PC – pasteurized milk cheese; PCLP – pasteurized milk cheese added with *L. plantarum* CNPC003.
a–d: average ± standard deviation with different lowercase letters within a row differ ($P < 0.05$) among cheese formulations, based on Tukey's test or Student's t-test.
A–D: average ± standard deviation with different uppercase letters within a column differ ($P < 0.05$) among days of ripening, based on Tukey's test or Student's t-test.
[a] Total dry extract < LD: below limit of detection.
[b] Permitted variation ranges, as outlined in the technical regulation for cheeses with medium and high moisture content, like coalho cheese (Brazil, 1996), are 36%–54.9%.
[c] Permitted variation ranges for Fat in Total Solids (FTS), as outlined in the technical regulation of identity and quality for coalho cheese (Brazil, 2001), are 35–60%.

the end of ripening, highlighting the efficacy of the food matrix in mechanically shielding these bacteria against adverse conditions throughout the ripening process.

3.2. Technological and physicochemical characteristics of goat coalho cheese

Table 2 shows the technological and physicochemical characteristics of the examined goat coalho cheeses, while Table 3 shows the instrumental texture parameters.

Goat coalho cheese samples had similar yields ($P \geq 0.05$), ranging from 157 to 166 g L^{-1} of milk. The syneresis, i.e., whey expulsion from the casein clot, increased during ripening in all goat coalho cheese samples ($P < 0.05$), especially in PC (from 1.50 to 7.76 g 100 g^{-1}) and RCLP (from 0.93 to 8.49 g 100 g^{-1}).

Cheese samples had medium to high moisture content (44.8–52.8 g 100 g^{-1}), consistent with expectations for coalho cheese, characterized by a cooked or semi-cooked curd, and classified as a semi-hard cheese with medium (36%–45.9%) to high moisture (46%–54.9%) (Brazil, 1996). Goat coalho cheeses made with raw milk had a lower moisture content ($P < 0.05$). Despite the increase in syneresis during ripening, the moisture content of goat coalho cheeses remained unchanged until 60 days of ripening ($P \geq 0.05$).

Lactose content decreased ($P < 0.05$) during ripening, especially in coalho cheese made with raw goat milk, which had values below the detection limit from day 20 of ripening onward. This behavior is expected during the storage or ripening of fermented dairy products when an increase in lactose metabolism into lactic acid commonly occurs. Natural microbiota, starter, and/or probiotic cultures generally use sugars in their metabolism to produce lactic acid as one of the main end-products (Costa, da Silva Frasao, da Costa Lima, Rodrigues, & Junior, 2016). Higher acidity in lactic acid at 60 days of ripening may have contributed to making cheeses safe for consumption, with low counts of the measured microorganisms (Table 1). The decrease in lactose content could be considered a positive point since goat coalho cheese could be better accepted and digested by individuals with lactose intolerance (Verruck et al., 2019).

An increase in acidity with a concomitant reduction in pH ($P < 0.05$) was observed during the 60 days of ripening, especially in cheeses made with raw milk and added with *L. plantarum* CNPC003 (RCLP). This can be explained by a more intense fermentation in these products, mainly due to lactose fermentation, caused by the symbiotic interaction of the complex and natural microbiota of raw milk, which, together with the added culture, favor the formation of organic acids (Arias-Roth, 2022). This behavior probably impacted cheese syneresis since the syneresis rate is directly related to acidity and, therefore, inversely related to pH. Because of the progressive increase in hydrogen ion concentration during acidification, the repulsive forces decrease, and casein micelles aggregate, producing whey expulsion and increased syneresis in cheese (Oliveira et al., 2012).

At 60 days of ripening, ash (minerals), NaCl, and protein contents did not differ among the examined goat coalho cheese samples ($P \geq 0.05$). Fat content in cheese samples varied from 18.9 to 25.2 g 100 g^{-1} (39.1% to 48.7% of fat in total solids – FTS), agreeing with the technical regulation of identity and quality for coalho cheese (Brazil, 2001), where FTS must be between 35% and 60%.

As for instrumental texture (Table 3), the hardness in cheeses made with raw milk (RC and RCLP) increased during ripening, while decreased in cheeses made with pasteurized milk (PC and PCLP) ($P < 0.05$). At 60 days of ripening, RC and RCLP had higher hardness ($P < 0.05$) than PC and PCLP. The hardness of cheeses is normally inversely correlated to moisture content, which may be related to the composition of raw milk native microbiota and, consequently, to organic acids production by these microorganisms, causing increased syneresis and decreased moisture content (Jia et al., 2021). This occurs due to the link between the rate and level of acidification and the demineralization of casein micelles,

Table 3
Texture profile of goat coalho cheeses made with raw or pasteurized milk and added or not with *L. plantarum* CNPC003 during 60 days of ripening (10 ± 1 °C).

Parameters	Days	Cheeses			
		RC	RCLP	PC	PCLP
Hardness (N)	1	55.8 ± 0.36^{aB}	51.9 ± 4.37^{aC}	35.7 ± 0.47^{bA}	55.1 ± 1.29^{aA}
	20	56.3 ± 3.18^{abB}	65.0 ± 2.57^{aB}	37.7 ± 4.33^{cA}	46.7 ± 3.47^{bcA}
	40	64.3 ± 4.21^{bAB}	76.6 ± 4.43^{aA}	33.7 ± 2.60^{cAB}	31.5 ± 4.41^{cB}
	60	71.6 ± 7.60^{aA}	71.1 ± 0.69^{aAB}	24.4 ± 5.04^{cB}	35.4 ± 0.91^{bB}
Adhesiveness (mJ)	1	0.90 ± 0.01^{aB}	1.05 ± 0.21^{aA}	0.95 ± 0.64^{aB}	1.45 ± 0.21^{aC}
	20	5.40 ± 2.83^{bA}	1.20 ± 0.42^{cA}	3.00 ± 1.98^{bcAB}	10.3 ± 2.97^{aAB}
	40	3.85 ± 0.07^{bcAB}	0.60 ± 0.14^{cA}	5.15 ± 0.35^{bA}	12.9 ± 1.49^{aA}
	60	0.75 ± 0.07^{bB}	0.35 ± 0.07^{bA}	1.60 ± 0.01^{bAB}	9.15 ± 1.06^{aB}
Springiness (mm)	1	8.60 ± 0.23^{aA}	9.19 ± 0.42^{aA}	8.59 ± 0.01^{aA}	8.83 ± 0.23^{aA}
	20	8.71 ± 0.04^{aA}	0.38 ± 0.04^{bC}	8.41 ± 0.26^{aA}	8.20 ± 0.12^{aAB}
	40	7.31 ± 0.33^{bB}	3.03 ± 0.17^{cB}	8.23 ± 0.09^{aAB}	7.46 ± 0.23^{bBC}
	60	4.39 ± 0.23^{bC}	2.99 ± 0.29^{cB}	7.58 ± 0.42^{aB}	7.06 ± 0.49^{aC}
Cohesiveness	1	0.41 ± 0.02^{bA}	0.60 ± 0.02^{aA}	0.50 ± 0.02^{abA}	0.57 ± 0.01^{aA}
	20	0.41 ± 0.04^{bA}	0.09 ± 0.01^{cB}	0.50 ± 0.08^{abA}	0.56 ± 0.08^{aA}
	40	0.26 ± 0.05^{bB}	0.08 ± 0.01^{cB}	0.50 ± 0.04^{aA}	0.53 ± 0.04^{aA}
	60	0.11 ± 0.01^{bC}	0.08 ± 0.01^{bB}	0.38 ± 0.06^{aA}	0.46 ± 0.07^{aA}
Gumminess (N)	1	22.4 ± 1.29^{bA}	30.9 ± 3.73^{aA}	17.7 ± 0.81^{bA}	31.2 ± 1.56^{aA}
	20	23.1 ± 0.83^{abA}	6.08 ± 1.32^{cB}	17.5 ± 0.54^{bA}	25.9 ± 5.57^{aA}
	40	16.2 ± 2.02^{aB}	5.75 ± 0.20^{bB}	16.7 ± 0.16^{aA}	16.5 ± 1.15^{aB}
	60	7.73 ± 0.31^{bC}	5.12 ± 0.52^{bB}	8.91 ± 0.28^{bB}	16.2 ± 1.94^{aB}
Chewiness (mJ)	1	200 ± 5.45^{bA}	268 ± 23.7^{aA}	151 ± 6.93^{cA}	268 ± 10.4^{aA}
	20	200 ± 6.44^{aA}	23.9 ± 6.22^{cB}	154 ± 10.3^{bA}	196 ± 24.4^{aB}
	40	125 ± 10.0^{aB}	17.4 ± 0.42^{bB}	137 ± 0.14^{aA}	122 ± 4.67^{aC}
	60	33.9 ± 0.42^{cC}	15.4 ± 3.04^{cB}	67.5 ± 1.70^{bB}	107 ± 10.8^{aC}

Cheeses: RC – raw milk cheese; RCLP – raw milk cheese added with *L. plantarum* CNPC003; PC – pasteurized milk cheese; PCLP – pasteurized milk cheese added with *L. plantarum* CNPC003.
a–c: average ± standard deviation with different lowercase letters within a row differ ($P < 0.05$) among cheese formulations, based on Tukey's test.
A–C: average ± standard deviation with different uppercase letters within a column differ ($P < 0.05$) among days of ripening, based on Tukey's test.

contributing to a lower whey retention capacity, increased syneresis, greater aggregation, compaction of casein micelles, and, consequently, harder texture in cheeses. Since pasteurization causes changes in milk, such as inactivation of native milk enzymes, elimination of milk natural microbiota, low acidity, slight denaturation of whey proteins, and changes in starter bacteria activity, the cheese characteristics (e.g., proteolysis and texture) are also altered and make the cheese softer (Natrella et al., 2023). In fact, cheeses made with raw milk (RC and RCLP) had higher acidity, lower moisture content, and higher hardness at 60 days of ripening ($P < 0.05$). Among the measured texture parameters, hardness was the most important, as cheeses with a brittle texture are usually less accepted by consumers, which was not observed in this study.

PCLP showed an increase in adhesiveness, which refers to the force needed to remove the material from the palate while chewing (sticky) (Poltórak et al., 2015), during ripening, and it had the highest adhesiveness (9.15 ± 1.06 mJ) at 60 days of ripening compared to other examined cheese samples ($P < 0.05$). The examined goat coalho cheese samples did not differ in springiness (ranging between 8.59 and 9.19 mm) on the first day of ripening, which was reduced during ripening ($P < 0.05$). Springiness is defined as the rate at which the deformed sample goes back to its undeformed condition after force has been removed (Faber, Jaishankar & McKinley, 2017), and its decrease is linked to reduced viscoelasticity in cheeses. However, cheeses made with pasteurized milk (PC and PCLP) had higher springiness ($P < 0.05$) when compared to cheeses made with raw milk (RC and RCLP) at 60 days of ripening.

Goat coalho cheeses made with raw milk (RC and RCLP) had a reduction in cohesiveness ($P < 0.05$) at 60 days of ripening, which is linked to the extent of deformation after the sample is compressed (Poltórak et al., 2015), while cheeses made with pasteurized milk (PC and PCLP) did not differ ($P \geq 0.05$). All goat coalho cheese samples had a reduction in gumminess and chewiness at 60 days of ripening, with PCLP having the greatest gumminess and chewiness ($P < 0.05$) (16.2 N and 107 mJ, respectively). PC and PCLP were softer at 60 days of ripening ($P < 0.05$) and had higher chewability than other cheese samples. Pasteurization can promote milk protein aggregation and the formation of firmer gel (Delgado, Da Silva Frasao, Da Costa, & Junior, 2017). In addition, the type of probiotic used for cheese production can affect the product texture. *L. plantarum* CNPC003 produces exopolysaccharides (EPS) that can affect cheese texture due to its ability to increase viscosity and modify gel strength, as well as to prevent syneresis and gel fracture (Bomfim et al., 2020; Delgado et al., 2017).

3.3. Protein, amino acidic, and peptide profile of goat coalho cheese

3.3.1. Soluble protein

Regarding soluble protein content (Table S3), no differences were found among the examined goat coalho cheese samples on the first day of ripening ($P \geq 0.05$). However, the soluble protein content increased in all cheese samples at 60 days of ripening, especially in RC, where the proteolytic process was more intense during ripening. This result may be related to the gradual increase in casein degradation, releasing soluble peptides expressed in the extent and depth of proteolysis indexes (Fialho et al., 2018).

3.3.2. Peptides identification by SDS-PAGE electrophoresis

An electrophoretic profile of goat coalho cheese samples at 60 days of ripening showed the presence of bands varying in size between 15 and 60 kDa, but the protein profile did not differ among the examined samples, agreeing with the results of previous studies (Barac et al., 2019; Özcan Yardım & Durak, 2023).

A band around 30 kDa was detected (Fig. 1), which was correlated to β-casein. The strong intensity of this band in the electrophoresis gel indicates that this is the protein in the largest quantity in the samples. β-Casein fraction is the main group of proteins in coalho cheese and an important fraction factor for cheese formation and solidification.

It was also possible to detect a less intense band around 25 kDa corresponding to α-s1 casein. A major degradation of casein during the ripening of goat cheeses could be linked to its lighter pattern (Uzkuç & Yüceer, 2023). In addition, the presence of bands smaller than 20 kDa in all goat coalho cheese samples at 60 days of ripening indicates the presence of peptides produced due to proteolysis caused by the enzyme system and the action of the added culture (Hao, Xia, Wang, Zhang, & Liu, 2023).

3.3.3. Peptides identification by advanced mass spectrometry

Table S4 shows the 10 most abundant proteins of each examined goat coalho cheese sample at 60 days of ripening. The number of identified peptides/spectra that may be related to the protein content in the cheese samples is also presented. In addition, the

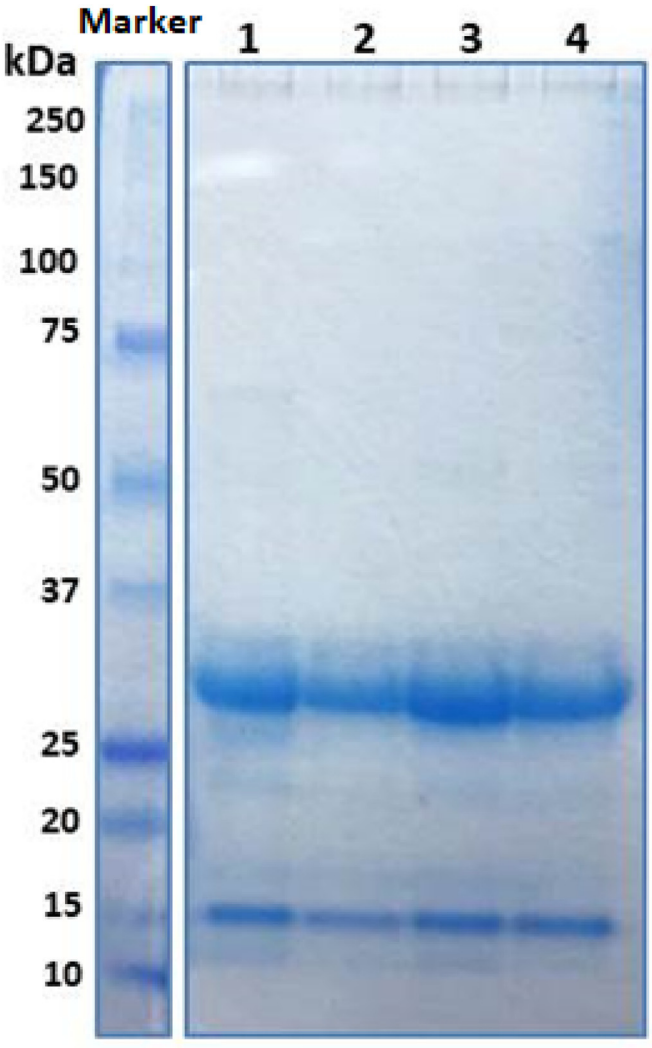

Fig. 1. SDS-PAGE electrophoresis of proteins in goat coalho cheese made with raw or pasteurized milk and with or not the addition of *Lactiplantibacillus plantarum* CNPC003 at 60 days of ripening (10 ± 1 °C). 1: RC – raw milk cheese; 2: RCLP – raw milk cheese with *L. plantarum* CNPC003; 3: PC – pasteurized milk cheese; 4: PCLP – pasteurized milk cheese with *L. plantarum* CNPC003.

percentage of each protein, estimated by the area of the obtained peaks, was obtained.

The proteome of goat milk is complex and diverse since it involves many proteins, such as caseins, whey proteins, immunoglobulins, lactalbumin, and lactoferrin. However, there are numerous groups of proteins present in lower concentrations, as well as post-translational modifications and genetic variations that can be visualized using mass spectrometry analysis (Verma, Dige, Gautam, De, & Rout, 2020).

Several proteins were identified in the goat coalho cheese samples (Table S5), indicating the total protein content in each one. As expected, casein was the major protein in all cheese samples (although this band was cropped and excluded from the analysis). PC had a higher total number of proteins compared to other cheese samples. This can be explained by the heat treatment applied to the milk used for cheese production, causing a greater opening of the protein chain, followed by hydrolysis through the action of rennet (chymosin enzyme).

It is important to highlight that the increase in peptides release in cheeses is directly related to the extent of the proteolysis index (Bezerra et al., 2016), and, in fact, PC had a higher extension index compared to other cheese samples (Fig. 2). Some proteins identified in the examined goat coalho cheese samples (Table S4) are normally considered contaminants by the analytical method used, such as trypsin (added during digestion) and keratins (common contaminants in sample preparation).

α-S1, α-S2, beta-casein, kappa casein, cadherin, and butyrophilin (proteins from the fat globule membrane) were the proteins present in goat coalho cheese samples, producing the majority of the peptides (Table S4). RC had the highest number of peptides from these proteins (1006 in total), and beta-casein accounted for 1/3 of the peptides. Since this protein was present in the greatest quantity (Fig. 1), it was responsible for producing most peptides, except for RCLP.

According to the bioactivity score (>0.5), 30 potential bioactive peptides (Table 4) could be listed, considering that the higher the score, the greater the possibility of this peptide performing biological activities. All these sequences had 20 or fewer amino acid residues and could be classified as bioactive peptides. GPFPLL,

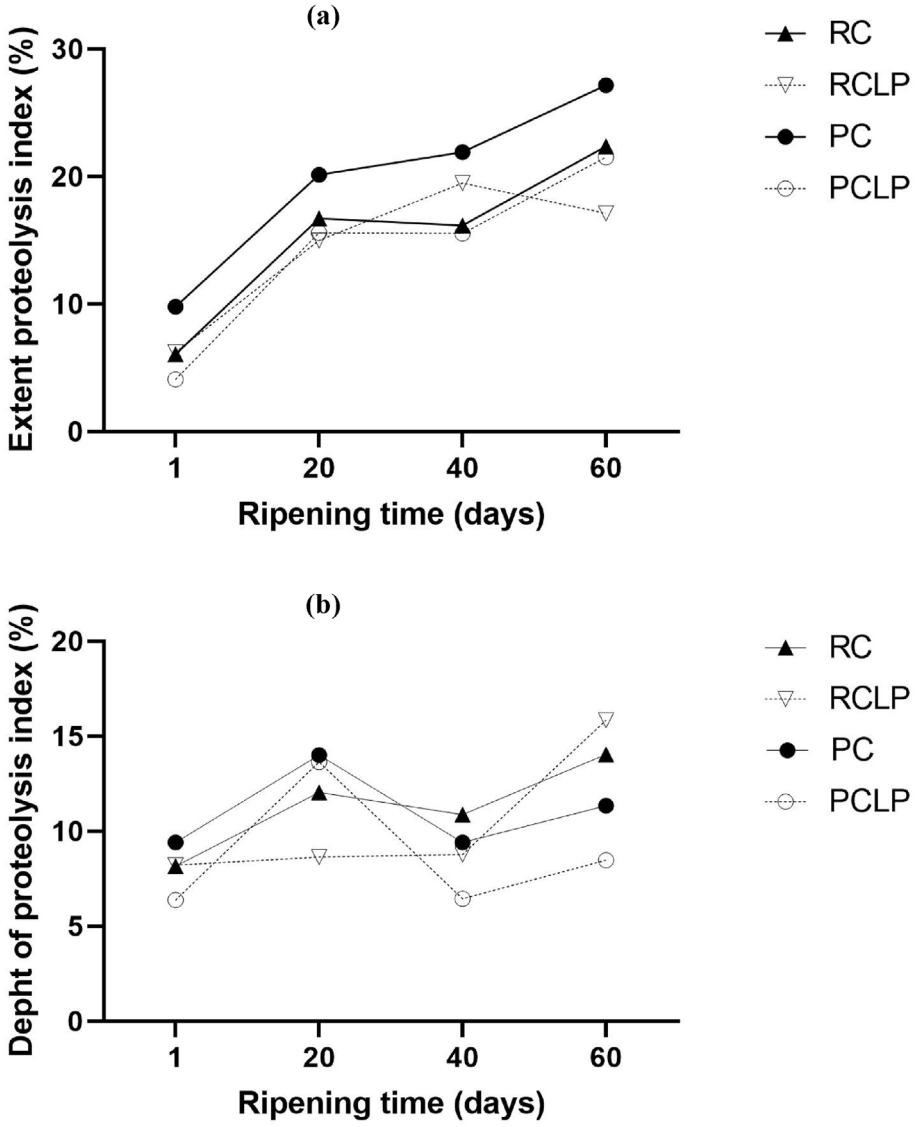

Fig. 2. Extent (a) and depth (b) of proteolysis indexes in goat coalho cheese made with raw or pasteurized milk and with or not the addition of *Lactiplantibacillus plantarum* CNPC003 during 60 days of ripening (10 ± 1 °C). Cheeses: RC – raw milk cheese; RCLP – raw milk cheese with *L. plantarum* CNPC003; PC – pasteurized milk cheese; PCLP – pasteurized milk cheese with *L. plantarum* CNPC003.

Table 4
Potential bioactive peptides found in goat coalho cheeses made with raw or pasteurized milk and added or not with *Lactiplantibacillus plantarum* CNPC003 after 60 days of ripening (10 ± 1 °C), according to the bioactivity score (>0.5).

Sequence	PepRank	Protein	Fragment	Length	Mass	P.i	Hydrophobicity Kcal mol^{-1}	Net charge at pH 7	Boman Index Kcal mol^{-1}	GRAVY	SAMPLE
GPFPLL	0.967691	Beta-casein	216–221	6	642,373	5.58	5.12	0	1.43	1.13	RCLP/PC/PCLP/RC
FDSWPCVMGR	0.956289	Butyrophilin	341–350	10	1196,509	6.07	10.2	0	1.31	−0.03	RCLP/PC/PCLP/RC
FYPQLFR	0.952151	Alpha-S1-casein	160–166	7	969,5058	9.69	5.24	1	1.38	−0.21	RCLP/PC/PCLP
SWFPYYAR	0.936327	Lactadherin	304–311	8	1088,507	9.44	5.59	1	1.43	−0.73	PC/PCLP
LEQLLR	0.93075	Alpha-S1-casein	110–115	6	770,4637	6.84	10.4	0	2.08	−0.02	RC
YFYPQLFR	0.93075	Alpha-S1-casein	159–166	8	1132,569	9.44	4.53	1	1.23	−0.35	RCLP/PC/PCLP
AYFYPQLFR	0.930466	Alpha-S1-casein	158–166	9	1203,606	9.7	5.03	1	0.89	−0.11	RCLP/PC/PCLP/RC
SAFSWFPYYAR	0.928479	Lactadherin	301–321	11	1393,644	9.44	4.84	1	0.91	−0.18	PC/PCLP/RC
FAWPQYLK	0.906686	Alpha-S2-casein	190–197	8	1051,548	9.59	6.35	1	−0.1	−0.35	RCLP/RC
LAYFYPQLFR	0.885723	Alpha-S1-casein	157–166	10	1316,69	9.65	3.78	1	0.31	0.28	RCLP/PC/PCLP
LSPEVPNENLLR	0.885723	Alpha-S1-casein	26–37	12	1379,739	4.08	15.2	−1	2.2	−0.58	RC
YLGYLEQLLRL	0.872773	Alpha-S1-casein	106–116	11	1379,779	6.56	7.59	0	0.18	0.41	RC
GPFPILV	0.869285	Beta-casein	216–222	7	741,4412	5.64	4.79	0	−2.54	1.67	RCLP/PC/PCLP/RC
GYWALTPLR	0.862325	Butyrophilin	402–410	9	1075,58	9.93	6.45	1	0.3	0.00	RCLP/PC/PCLP/RC
GPVRGPFPILV	0.860405	Beta-casein	212–222	11	1150,684	11.13	7.43	1	−0.71	0.85	PCLP
APFPEVFR	0.858066	Alpha-S1-casein	41–48	8	961,5007	6.89	10.2	0	1.24	0.05	RCLP/PC/PCLP/RC
GPPEPILAMLGEDAELPCR	0.853296	Butyrophilin	33–51	19	2006,977	3.56	22.5	−3	0.8	−0.09	RC/PCLP
DMPIQAFLL	0.84613	Beta-casein	197–205	9	1046,545	3.12	6.95	−1	−0.84	1.11	RCLP/PC/PCLP/RC
PLPLAGPPRR	0.83541	Butyrophilin	412–421	10	1072,649	12.5	11.2	2	1.72	−0.64	PCLP/RC
SLVYPF	0.83317	Beta-casein	72–77	6	724,3784	5.38	4.37	0	−1.4	1.18	RCLP/RC
YQEPVLGPVRGPFPIL	0.836379	Beta-casein	206–221	16	1780,985	6.58	10.0	0	−0.01	0.21	RCLP/PC/RC
EPVLGPVRGPFPILV	0.831327	Beta-casein	208–222	15	1588,931	6.73	9.49	0	−0.66	0.82	PC
PVLGPVRGPFPILV	0.830998	Beta-casein	209–222	14	1459,889	11.56	5.86	1	−1.2	1.13	PC
FLLYQEPVLGPVRGPFPILV	0.823413	Beta-casein	203–222	20	2253,289	6.62	5.34	0	−0.85	0.90	PC
YQEPVLGPVRGPFPILV	0.814751	Beta-casein	206–222	17	1880,053	6.58	9.55	0	−0.25	0.44	RCLP/PC
LPLAGPPRR	0.811426	Butyrophilin	413–421	9	975,5962	12.49	11.1	2	1.91	−0.53	RCLP/PCLP/RC
TWGLSAFSWFPYYAR	0.809312	Lacthaderina	307–311	15	1850,875	9.39	2.9	1	0.29	−0.01	RC
DMPIQAFL	0.80528	Beta-casein	197–204	8	933,4615	3.12	8.2	−1	−0.33	0.78	RCLP/PC/PCLP/RC
HPHPHLSF	0.79667	K-casein	110–117	8	970,4761	7.93	12.6	0	1.18	−0.88	RCLP/PC/PCLP/RC
LYQEPVLGPVRGPFPIL	0.794885	Beta-casein	205–221	17	1894,068	6.77	8.76	0	−0.3	0.42	RCLP/PC

Cheeses: RC – raw milk cheese; RCLP – raw milk cheese added with *L. plantarum* CNPC003; PC – pasteurized milk cheese; PCLP – pasteurized milk cheese added with *L. plantarum* CNPC003. Amino acids are represented by the one-letter code: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; Y = Tyr. The probability of bioactivity (%) was assessed using the PeptideRanker bioinformatics tool.

FDSWPCVMGR, FYPQLFR, SWFPYYAR, and LEQLLR were the sequences with the higher bioactivity scores, respectively.

Regarding the characteristics analyzed in the peptides, some particularities can be highlighted. The hydrophobicity values of the peptides ranged from 2.9 in the TWGLSAFSWFPYYAR peptide to 22.54 in the GPPEPILAMLGEDAELPCR sequence. This variation is directly related to the greater presence of polar amino acids, such as alanine, leucine, isoleucine, valine, and proline, or aromatic amino acids, such as phenylalanine, tryptophan, and methionine. Generally, the degree of hydrophobicity conferred by the amino acid sequence in the peptide is directly related to the biological activity score, which is a fundamental characteristic for expressing activities, such as antimicrobials, anticancer agents, antioxidants, and anti-inflammatory compounds, influencing the three-dimensional structure of peptides and enhancing the ability to penetrate cell membranes and interact with intracellular proteins.

Most of the sequences showed an isoelectric point at basic pH, which means that most of them assume positive charges in goat coalho cheese samples. LEQLLR was the sequence with the highest Boman index value. This value estimates a protein binding potential, and the higher it is, the more likely it is to bind to receptors and perform different functions. This sequence, containing 3 leucine in the molecule, also showed a high hydrophobicity (10.40 kcal mol^{-1}), with a predicted bioactivity probability of 81% by the peptide ranker. Meanwhile, "PLPLAGPPRR," with a hydrophobicity of 11.2 kcal mol^{-1} and BI of 1.72 kcal mol^{-1}, had its predicted probability at 84%, highlighting the relevance of hydrophobicity and amino acid sequence in bioactivity.

Biopep calculations for A value return a value corresponding to how often a matched fragment with biological activity is repeated in the peptide sequence. It was possible to list the 10 sequences that showed the greatest potential for inhibiting the angiotensin-converting enzyme (ACE) and the enzyme dipeptidyl peptidase IV (DPP IV) (Table S6). These activities have been frequently reported by other authors since are related to proteins present in cheese, such as beta and alpha casein (Khakhariya et al., 2023).

PC, PCLP and RCLP cheeses presented most sequences with potential for ACE inhibition, where the LAYFYPQLFR fragment originating from a-s1 casein proved to be the peptide with the greatest ACE inhibition potential, with similarity to a sequence (LAYFYPQL) previously identified (Moreno-Montoro et al., 2018). As for DPP IV inhibition, RC cheeses presented most sequences with this potential, and GPFPLL, a beta-casein fragment, was the sequence that returned the highest A value, and this is the one with the greatest potential to exert antidiabetic activities. A sequence partially similar to this was identified in Parmigiano Reggiano cheeses (Castellone et al., 2022). Fig. 3 shows a schematic drawing of the primary structure and 3D model of these peptides.

3.3.4. Total and free amino acids

The total amino acid contents of the different examined coalho cheese samples did not vary significantly (Table S7). In general, goat coalho cheeses proved to be a good source of protein, with the presence of all essential amino acids, without considerable changes over the measured ripening time.

Regarding free amino acids, casein hydrolysis by coagulants, occurring during the first cheese ripening stage, leads to the production of high-molecular-weight peptides. Microbial enzymes break these peptides into smaller peptides and free amino acids, so

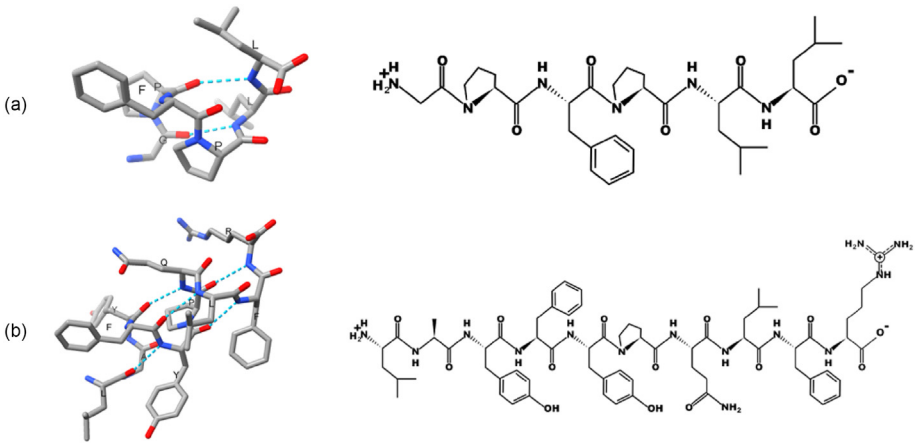

Fig. 3. Schematic representations of the structure of the GPFPL (a) and LAYFYPQLFR (b) peptides.

the cheese-free amino acid profile is linked to microorganisms participating in cheese ripening (Atanasova et al., 2021).

The addition of *L. plantarum* CNPC003 and ripening time caused a greater release of free amino acids in goat coalho cheese, especially in RCLP, which had free amino acids content of 1583 ± 3.95 mg 100 g^{-1} at 60 days of ripening (Table 5). Agreeing with these results, RCLP was the sample with the highest rate in depth of proteolysis at 60 days of ripening (Fig. 2b), suggesting that the use of raw milk in cheese processing, as a source of native LAB, together with the addition of *L. plantarum* CNPC003, contributed to

Table 5
Free amino acids found in goat coalho cheeses made with raw or pasteurized milk added or not with *Lactiplantibacillus plantarum* CNPC003 at day 1 and 60 of ripening (10 ± 1 °C).

Free amino acids (mg 100 g^{-1})	Days	Cheeses			
		RC	RCLP	PC	PCLP
Histidine	1	76.2 ± 1.15^{aA}	<LD	60.9 ± 0.09^{b}	56.9 ± 1.40^{cB}
	60	59.3 ± 0.84^{bB}	72.3 ± 0.62^{a}	<LD	60.2 ± 0.20^{bA}
Threonine	1	28.6 ± 0.36^{aA}	<LD	19.8 ± 0.13^{bA}	<LD
	60	23.8 ± 0.02^{cB}	30.6 ± 0.06^{b}	20.2 ± 0.08^{dA}	72.6 ± 0.06^{a}
Tyrosine	1	59.4 ± 0.52^{aB}	40.9 ± 0.93^{bB}	40.4 ± 0.86^{bB}	35.6 ± 0.03^{cB}
	60	62.9 ± 1.18^{aA}	56.3 ± 0.55^{bA}	45.3 ± 0.46^{cA}	44.6 ± 0.09^{cA}
Valine	1	8.56 ± 0.07^{aB}	<LD	<LD	<LD
	60	77.3 ± 1.27^{bA}	135 ± 0.13^{a}	11.3 ± 0.26^{c}	78.1 ± 0.21^{b}
Methionine	1	<LD	<LD	<LD	<LD
	60	15.9 ± 0.16^{b}	31.7 ± 0.04^{a}	1.20 ± 0.13^{d}	6.60 ± 0.18^{c}
Cysteine	1	27.1 ± 5.32^{cA}	29.8 ± 1.69^{bcA}	36.2 ± 1.05^{bA}	43.2 ± 1.53^{aA}
	60	25.6 ± 0.04^{bA}	29.8 ± 1.12^{abA}	35.7 ± 1.02^{aA}	33.2 ± 0.91^{aB}
Isoleucine	1	6.43 ± 0.13^{aB}	1.52 ± 0.02^{abB}	0.96 ± 0.07^{bA}	<LD
	60	37.1 ± 0.09^{cA}	58.7 ± 2.02^{bA}	0.86 ± 1.21^{dA}	109 ± 4.08^{a}
Leucine	1	120 ± 11.6^{dB}	251 ± 6.75^{aB}	178 ± 6.80^{cA}	212 ± 5.50^{bA}
	60	272 ± 9.71^{bA}	400 ± 9.76^{aA}	169 ± 8.08^{cA}	133 ± 0.07^{dB}
Phenylalanine	1	21.9 ± 0.86^{aB}	<LD	<LD	5.24 ± 0.14^{bB}
	60	109 ± 0.52^{bA}	122 ± 4.85^{a}	14.6 ± 0.26^{d}	65.6 ± 0.19^{cA}
Lysine	1	5.97 ± 0.02^{aB}	<LD	<LD	<LD
	60	21.6 ± 0.09^{cA}	64.7 ± 0.41^{a}	3.53 ± 0.09^{d}	22.8 ± 0.60^{b}
Tryptophan	1	68.0 ± 1.60^{aB}	<LD	<LD	7.41 ± 0.35^{bB}
	60	89.9 ± 0.46^{bA}	127 ± 1.67^{a}	<LD	85.7 ± 1.17^{cA}
Aspartic acid	1	11.6 ± 0.69^{aB}	6.16 ± 0.13^{bA}	6.51 ± 0.11^{bA}	<LD
	60	37.7 ± 0.04^{aA}	6.64 ± 0.79^{bA}	5.97 ± 0.07^{bA}	0.41 ± 0.58^{c}
Glutamic acid	1	7.45 ± 0.13^{aB}	5.80 ± 0.06^{bB}	1.91 ± 0.06^{dB}	5.01 ± 0.01^{cB}
	60	21.7 ± 0.08^{cA}	72.5 ± 0.40^{bA}	9.78 ± 0.16^{dA}	91.4 ± 0.25^{aA}
Serine	1	68.9 ± 2.61^{aA}	2.33 ± 0.36^{bB}	5.71 ± 0.01^{bA}	2.65 ± 0.03^{bB}
	60	10.3 ± 0.16^{bB}	25.4 ± 0.18^{aA}	7.53 ± 0.01^{bA}	7.76 ± 1.56^{bA}
Glycine	1	36.7 ± 0.47^{aA}	9.10 ± 0.01^{bB}	7.55 ± 0.01^{bA}	7.74 ± 0.01^{bA}
	60	16.3 ± 0.01^{bB}	24.2 ± 1.27^{aA}	7.96 ± 0.11^{cA}	6.58 ± 0.62^{cA}
Arginine	1	3.73 ± 0.23^{bB}	3.38 ± 0.04^{cB}	3.93 ± 0.01^{bA}	<LD
	60	110 ± 1.20^{aA}	188 ± 2.40^{bA}	3.84 ± 0.03^{cA}	3.00 ± 0.13^{d}
Alanine	1	17.9 ± 0.11^{aB}	5.71 ± 0.11^{bB}	2.10 ± 0.04^{dB}	5.02 ± 0.06^{c}
	60	29.8 ± 0.23^{bA}	68.3 ± 0.30^{aA}	18.0 ± 0.13^{cA}	<LD
Proline	1	3.82 ± 0.21^{aB}	<LD	<LD	<LD
	60	31.9 ± 0.47^{cA}	68.2 ± 0.46^{a}	28.9 ± 0.01^{d}	53.5 ± 0.16^{b}
Total	1	572 ± 13.9^{aB}	356 ± 3.51^{bB}	364 ± 4.86^{bA}	380 ± 5.98^{bB}
	60	1054 ± 10.9^{bA}	1583 ± 3.95^{aA}	384 ± 7.50^{dA}	875 ± 5.81^{cA}

<LD: below limit of detection. Cheeses: RC – raw milk cheese; RCLP – raw milk cheese added with *L. plantarum* CNPC003; PC – pasteurized milk cheese; PCLP – pasteurized milk cheese added with *L. plantarum* CNPC003. a–d: average ± standard deviation with different lowercase letters within a row differ ($P < 0.05$) among cheese formulations, based on Tukey's test or Student's t-test. A–B: average ± standard deviation with different uppercase letters within a column differ ($P < 0.05$) among days of ripening, based on Student's t-test.

an increase in amino acids and low molecular weight peptides release. On the other hand, cheeses made with pasteurized goat milk had lower levels of free amino acids (384 ± 7.50 to 875 ± 5.81 mg per 100 g) and shallower depths of proteolysis (Fig. 2b), reinforcing the impact of pasteurization on reducing the microbiota of raw milk, thereby directly affecting these characteristics in cheeses.

Concentrations of methionine, leucine, and glutamic acid are commonly considered indicators of protein hydrolysis degree in cheese (Suzuki-Iwashima, Matsuura, Iwasawa, & Shiota, 2020). The contents of these amino acids overall increased ($P < 0.05$) during ripening, especially in goat coalho cheeses made with raw milk.

It is noteworthy that amino acids play an important role in human health, such as anti-inflammatory activity, synthesis of antioxidant enzymes, neurological and behavioral functions, and immune response modulatory effects. As an example, arginine, detected in higher concentrations in RCLP (188 ± 2.40 mg 100 g^{-1}) at 60 days of ripening, helps to regulate blood pressure and improve physical performance, as it is an oxide nitric acid precursor (Apolzan et al., 2022). Proline, an amino acid that constitutes connective tissues and plays an important role in healing processes (Vettore, Westbrook, & Tennant, 2021), increased at 60 days of ripening, especially in RCLP, confirming a higher concentration of this amino acid at the end of the ripening period (68.2 ± 0.46 mg 100 g^{-1}) (Table 5).

Considering the large amount of amino acid, the examined goat coalho cheese samples, mainly RCLP, could serve as delivery vehicles for these compounds to the body, providing bioactive properties to the consumers.

3.3.5. Proteolysis

Proteolysis is an important event during cheese ripening, contributing to changes in sensory parameters, such as texture, and the development of suitable flavors. These changes result from degradation products, including peptides and free amino acids (Coelho et al., 2022). It is known that the extent of proteolysis is related to the activity of enzymes present in coagulants and microbial enzymes used in cheese manufacturing, which degrade proteins into high molecular-weight peptides (Morais et al., 2022). Depth of proteolysis is related to the presence of substances of medium and low molecular weight and results from the activity of proteolytic enzymes, such as proteinases and peptidases from LAB, degrading casein peptides into smaller peptides and free amino acids, and constitute the fraction soluble in 12% TCA (Bezerra et al., 2016).

All examined goat coalho cheese samples had increased proteolysis rates (mainly in extension) during the 60 days of ripening, which also corroborates the increase in soluble protein (Table S3) and free amino acids (Table 5). PC showed a higher extent of proteolysis index ($P < 0.05$) at 60 days of ripening, indicating a greater amount of high molecular weight peptides in cheeses made with pasteurized milk and ripened until 60 days and, possibly justifying the higher amount of proteins found therein (Table S5), considering a lower action of proteinases in the release of amino acids (Table 5). Furthermore, RCLP showed a higher rate of depth of proteolysis at 60 days of ripening, justifying the higher amount of free amino acids (Table 5) and lower content of proteins (Table S5). Enzymes are more likely to influence proteolysis in cheeses than the LAB used during processing. In fact, changes in proteolysis are mainly catalyzed by residual chymosin and, to a lesser extent, by other proteases present in the curd, such as plasmin, or proteases from the cell envelopes of LAB cultures used in processing (Oliveira et al., 2012). Corroborating this behavior, a higher percentage of extensive proteolysis (>20%), especially in cheeses without *L. plantarum* CNPC003 (PC and RC) was found at 60 days of ripening, while values <20% for depth of proteolysis were found for all examined goat coalho cheese samples (Fig. 2).

3.3.6. Principal component analysis (PCA)

A principal component analysis was performed to verify the correlations between examined goat coalho cheese samples at 60 days of ripening and their protein, peptide, and amino acid profiles. To this end, results of identifying and quantifying free amino acids, extent and depth of proteolysis index, total number of identified proteins, and potentially bioactive peptide sequences according to PepRanker bioactivity score (>0.5) were incorporated. For this analysis, ten peptide sequences identified and evaluated according to Biopep with the highest scores for ACE and DPP IV enzyme inhibition activity was classified according to their presence and peak area in each cheese sample.

Fig. 4 shows that most free amino acids are in quadrants on the right side, where goat coalho cheese samples made with raw milk are also located, mostly near RCLP, except for cysteine. The same occurs with the DPI variable, which refers to the depth of proteolysis related to the action of proteinases and peptidases from LAB, which corroborates the extensive presence of amino acids in this region together with goat coalho cheese samples made with raw milk, especially RCLP, probably caused by the native microbiota together with the added *L. plantarum* CNPC003, producing low molecular weight peptides and amino acids (Bezerra et al., 2016).

On the other hand, the EPI variable is very close to PC, as well as the total identified proteins (TIP), in which PC had the highest concentration. This result validates that using pasteurized milk together with the non-addition of *L. plantarum* CNPC003 resulted in less protein breakdown, where the proteolytic action was mostly due to rennet addition during cheese production (Morais et al.,

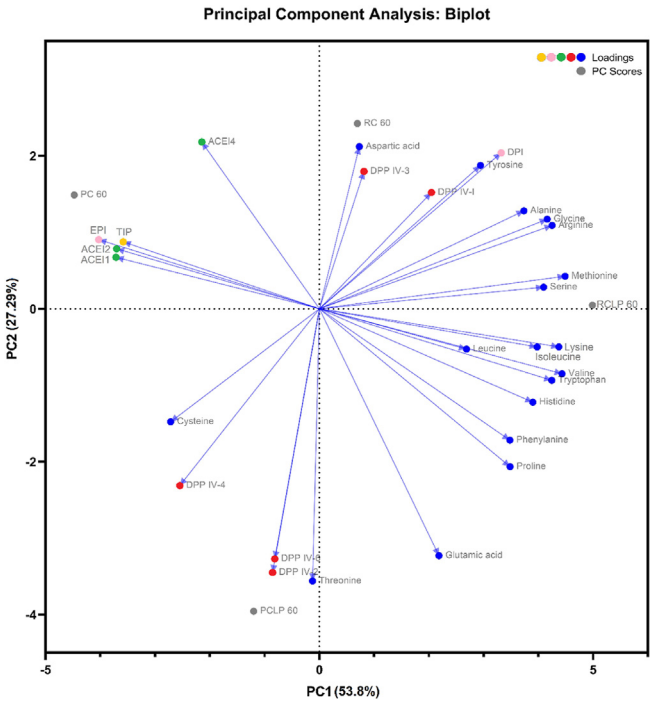

Fig. 4. Principal component analysis. ● Goat coalho cheese samples at 60 days of ripening (RC – raw milk cheese; RCLP – raw milk cheese with *L. plantarum* CNPC003; PC – pasteurized milk cheese; PCLP – pasteurized milk cheese with *L. plantarum* CNPC003); ● Free amino acids identified; ● DPP IV – dipeptidyl peptidase IV potentially inhibiting peptide sequences; ● ACEI – angiotensin-converting enzyme potentially inhibiting peptide sequences; ● TIP – total identified proteins; ● EPI – extent of proteolysis index; DPI – depth of proteolysis index.

2022). This behavior consequently resulted in a lower production of free amino acids, which are important components in volatile compound formation that play an important role in the flavor and cheese aroma, in addition to being precursors of bioactive compounds (Ayag, Dagdemir, & Hayaloglu, 2022).

Regarding the peptides with bioactive potential, it is possible to observe a grouping of peptide sequences with potential for ACE inhibition (ACEI1 – LAYFYPQLFR; ACEI2 – AYFYPQLFR; and ACEI4 – YFYPQLFR) close to PC, where all sequences were originated from the alpha-s1-casein protein. Regarding the selected sequences with the potential to inhibit the DPP-IV enzyme, three of these sequences (DPP IV-2 - SWFPYYAR; DPP IV-4 – SAFSWFPYYAR; and DPP IV-6 – LEQLLR) were closer to PCLP, and two of these sequences (DPP IV-1 – GPFPLL; and DPP IV-3 – FAWPQYLK) were close to RC and RCLP. The correlation between these points can be justified by a possible production of these peptides with the potential to inhibit DPP-IV by LAB from raw milk and the added *L. plantarum* CNPC003, which did not occur with PC.

4. Conclusion

In this study, it was verified for the first time that using raw or pasteurized milk and adding or not the potentially probiotic strain *L. plantarum* CNPC003 clearly impacts, in different ways, the microbiological, technological, and physicochemical parameters and protein, amino acid, and peptide profile of goat coalho cheeses during 60 days of ripening. At the end of the measured ripening period, the examined goat coalho cheeses were safe for consumption and effective matrices to carry and maintain high LAB counts (>8 log CFU g^{-1}). Cheeses made with raw milk (RC and RCLP) were harder and less chewy than cheeses made with pasteurized milk (PC and PCLP), which were softer and chewier at 60 days of ripening. All cheeses had an increase in proteolysis rates until 60 days of ripening, corroborating with the increase in soluble protein and free amino acids. PC showed a higher extension of proteolysis index at 60 days of ripening, justifying a higher amount of high molecular weight peptides, a higher number of identified proteins, and a lower amount of free amino acids. In turn, RCLP showed a higher depth of proteolysis index at 60 days of ripening, which could be linked to the higher amount of free amino acids and lower number of proteins at this ripening period. Among the 30 potentially bioactive peptides listed, all examined cheese samples presented peptide sequences with potential bioactivities, where sequences with potentially angiotensin-converting enzyme (ACE) inhibition activity were more prevalent in PC, while potentially dipeptidyl peptidase IV (DPP IV) inhibiting sequences were more prevalent in RC, PCLP, and RCLP. However, RCLP also stood out with the more pronounced presence of free amino acids, demonstrating its potential as a functional food with enhanced bioactive properties to benefit consumer health. Milk pasteurization and the addition of *L. plantarum* CNPC003 contributed to a differentiation in the protein, peptide, and amino acid profile of goat coalho cheese, being a biotechnological tool to be exploited for the functionalization and preservation of other types of cheeses.

Funding

This research was partially funded by CAPES (Brazil), grant number 001; by Fundação de Amparo à Pesquisa do Estado da Paraíba (FAPESQ, Brazil), grant number 1010/2021; by CNPq (Brazil), Process number 308253/2020-5; as well as by Fundação de Amparo à Pesquisa do Estado da Paraíba (FAPESQ, Brazil), grant number 946/2021; and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil), grant number 2019/23896-6.

Data availability

Data could be made available on request.

CRediT authorship contribution statement

Camila Neves Meireles Costa: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Julia Mariano Caju de Oliveira:** Writing – review & editing, Methodology, Formal analysis. **Angela Matilde da Silva Alves:** Methodology, Formal analysis. **Kaíque Yago Gervazio de Lima:** Methodology, Formal analysis. **Maria Isabel Ferreira Campos:** Methodology, Formal analysis. **Antônio Silvio do Egito:** Resources, Methodology, Formal analysis. **Karina Maria Olbrich dos Santos:** Resources, Methodology, Formal analysis. **Mônica Correia Gonçalves:** Methodology, Formal analysis. **Evandro Leite de Souza:** Writing – review & editing, Methodology, Formal analysis. **Maria Teresa Bertoldo Pacheco:** Resources, Methodology, Formal analysis. **Adriane Elisabete Costa Antunes:** Supervision, Methodology, Funding acquisition, Formal analysis. **Maria Elieidy Gomes de Oliveira:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

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.

Acknowledgments

The authors acknowledge Prof. Dr. Rita de Cássia R. do E. Queiroga (Federal University of Paraíba), who sadly died in 2021 because of SARS-Covid-19, and after 30 years of dedicated research focused on dairy products from goats and, latterly, donkeys' milk and cactus. The Prof[a] Rita Queiroga was at the forefront of the idealization of the research that generated the data presented in this manuscript, and in 2019 approved the granting of financial support to research projects in the Public Call FAPESP-FAPESQ 2019 - Fostering Collaborative Research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2024.105972.

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