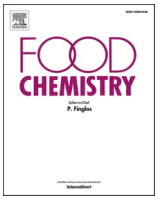
Food Chemistry 429 (2023) 136979

Contents lists available at ScienceDirect

Food Chemistry

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



The impact of heating and drying on protease activities of ruminant milk before and after *in vitro* infant digestion

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ARTICLE INFO

Keywords:
Ruminant milk
Proteases
Protein hydrolysis
Drying
Heating
Digestion

ABSTRACT

This study investigated the effect of heating (63°C/30 min or 75°C/15 s) and drying (spray-drying or freeze-drying) on plasmin, cathepsin D, and elastase activities in bovine, ovine, and caprine milk, compared to non-dried raw milk counterparts. Protease activities and protein hydrolysis were assessed before and after *in vitro* infant digestion with or without gastric and pancreatic enzymes. At 75°C/15 s, plasmin activity in caprine and ovine milk decreased (69–75%, $p<0.05$), while cathepsin D activity in spray-dried bovine milk heated increased (2.8-fold, $p<0.05$). Plasmin and cathepsin D activities increased (<1.2-fold, $p<0.05$) after *in vitro* digestion with pancreatin, regardless of milk species. Endogenous milk enzymes hydrolyzed more proteins than gastric enzymes during gastric digestion and contributed to small intestinal digestion. In summary, milk proteases remained active after processing with effects dependent on the species of milk, and they contributed to *in vitro* protein hydrolysis in the stomach and small intestine.

1. Introduction

Milk has diverse proteolytic enzymes (e.g., plasmin, cathepsin D, elastase) to cleave milk proteins into peptides and amino acids (Dallas et al., 2014). Proteases are part of a proteolytic system that consists of active enzymes (proteases), zymogens (inactive enzymes), inhibitors, and activators (Politis et al., 1993).

The most abundant protease in milk is plasmin, which exists primarily in its zymogen form, plasminogen, and can be converted into active plasmin by plasminogen activators (Grufferty & Fox, 1988). Plasmin and plasminogen are part of a complex system with also contain plasminogen activator inhibitors and plasmin inhibitors, which can affect plasminogen activators and plasmin, depending on the processing conditions. Plasmin preferably hydrolyses α_{s1}-, α_{s2}-, and β-caseins, and it is most active at pH 7.5 to 8 at 37°C (Prado et al., 2006). Cathepsin D is the second most abundant protease in milk and, like plasmin, is part of a complex system. Although the active form of cathepsin D is found in bovine milk, the predominant form of this enzyme in bovine milk is the inactive zymogen procathepsin D (Dallas et al., 2015). Cathepsin D shows the highest activity at pH 3.5 and 37°C, and it has a preference to hydrolyze α_{s1}-, α_{s2}-, β-, and κ-caseins, α-lactalbumin, and β-lactoglobulin (Gautam et al., 2023; Holton et al., 2014). Elastase is also present in bovine milk, but its concentration is lower compared to that of plasmin and cathepsin D (Albenzio et al., 2009). Elastase is a serine protease with a strong preference for hydrolyzing α_{s1}- and β-caseins (Considine et al., 1999), and its enzymatic activity is optimal at pH 7.5. Limited knowledge exists regarding other components of the elastase system beyond the active enzyme and its inhibitors (Considine et al., 1999).

The components of the proteolytic system are affected by many factors (e.g., temperature, pH), which suggests that selective proteolysis can be induced (Gan et al., 2019; Leite et al., 2021). Processing can affect the protein structures and physical characteristics of dairy products. Structural modifications of proteins can influence protease activation and inactivation and the susceptibility of milk proteins to both milk endogenous and gastric and small intestinal proteases. In addition, different conditions (e.g., pH and presence of digestive enzymes) exist

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https://doi.org/10.1016/j.foodchem.2023.136979
Received 30 January 2023; Received in revised form 20 July 2023; Accepted 21 July 2023
Available online 22 July 2023

between the gastric and small intestinal lumen that could affect the activity of milk proteases and their effects on protein digestion.

Most studies investigating the sensitivity of proteases to thermal treatment have been conducted with bovine milk (Anema, 2019; Stoeckel et al., 2016). For example, mild heat treatment (63°C/30 min) of bovine milk increased the activity of plasmin due to the inactivation of inhibitors and activation of plasminogen (Leite et al., 2021). However, little is known about the effect of heating and drying processes on the protease activities of ovine and caprine milk and its consequence on gastric and small intestinal protein digestion.

This study aimed to investigate the effect of heating (63°C/30 min or 75°C/15 s vs raw (control)) and drying (spray-drying or freeze-drying vs non-dried) processes on the activity of the three main endogenous proteases (plasmin, cathepsin D, and elastase) in bovine, ovine, and caprine milk. This study also aimed to determine the activity of plasmin and cathepsin D and the degree of protein hydrolysis of these milk after *in vitro* gastric (60 min) and small intestinal (60 min of gastric digestion plus 60 min of small intestinal digestion) digestion with or without digestive enzymes (pepsin, lipase, and pancreatin) mimicking infant digestion. Infant *in vitro* digestion conditions were chosen as bovine, ovine, and caprine milk are used to produce infant formulae. The hypotheses were that (i) the degree of protease activity in ruminant milk is influenced by heating and/or drying processing but dependent on the species of milk; and (ii) milk proteases after the processes remain active under *in vitro* gastric and small intestinal digestion conditions and contribute to protein hydrolysis.

2. Material and methods

The diagram of the experimental design of this study is shown in Supplementary Fig. 1.

2.1. Milk sampling and characterization

Fresh raw bovine milk was provided by Dairy 4 Farm at Massey University (Palmerston North, New Zealand), and ovine and caprine milk were supplied by local dairy farms (Palmerston North, New Zealand). For each species, three batches of milk were collected over three continuous days (i.e., n = 3 biological replicates). Raw ruminant milk samples were characterized for protein, fat, solids-not-fat, and total solids using a MilkoScan FT1 (Foss Electric, Hillerød, Denmark). The somatic cell count (SCC) was determined by the New Zealand Veterinary Pathology Laboratory.

On the same collection day, milk samples were skimmed at 50°C using a centrifugal separator (GEA Westfalia, Germany) and aliquoted and frozen at −80°C to avoid proteolysis during the storage. Raw skimmed ruminant milk samples were used as a control to compare the enzymes activities and degree of protein hydrolysis before and after heat treatments, drying processes and digestions. Sample analysis was done within two weeks.

2.2. Heat treatments

Raw skim milk was heat treated at 63°C for 30 min or 75°C for 15 s on the same collection day. These thermal conditions were chosen to represent conventional pasteurization using Low-Temperature Long-Time (63°C for 30 min) and High-Temperature Short-Time (75°C for 15 s) conditions. Ruminant milk samples were pasteurized at 63°C for 30 min using a thermostatically-controlled water bath with shaking (Leite et al., 2021) or at 75°C for 15 s using a pilot-plant-scale pasteurizer (Alpha Laval, Sweden). Heat treated milk samples were aliquoted and frozen at −80°C and analyzed within two weeks.

2.3. Drying processes

Raw and heat treated (63°C/30 min or 75°C/15 s) skimmed milk samples were dried using spray- or freeze-drying. For the spray-drying process, due to the limitation of processing one sample at a time, the raw milk sample was processed first, while the heat treated samples were stored in the refrigerator at 4°C, awaiting their turn to be dried. During each spray-drying cycle, a flask containing 2 L of the respective sample was placed in a water bath at temperature of 20°C. Raw and heat treated milk samples were dried and using a pilot spray-dryer (GEA, model Niro Mobile Minor, Denmark), with an inlet temperature of 185 ± 5°C and an outlet temperature of 80 ± 5°C. The feed rate was maintained at 20 mL/min using a peristaltic pump. For the freeze-drying process, samples (raw and heat treated) were transferred to plastic bags and placed on aluminum trays to freeze at −20°C for 24 h. Freeze-drying was carried out in a pilot-scale freeze-drier (Cuddon Freeze Dry, model FD18, New Zealand) at −1 mbar vacuum for 72 h with the condenser set up to −40°C. Dried milk samples were frozen at −20°C and analyzed within two weeks.

2.4. Simulated infant digestion

Dried milk samples were reconstituted to the original sample volume with Milli-Q water prior to measuring enzymatic activities or digestion. Then, an infant in vitro digestion of non-dried (raw (control) and heat treated (63°C/30 min or 75°C/15 s)) and dried (spray- and freeze-dried raw or heat treated) milk samples were performed based on a protocol described by Ménard et al. (2018).

The static in vitro digestion model was set up with and without digestive enzymes to mimic gastric (pepsin and lipase) and small intestinal (pancreatin) conditions in the infant. The oral phase was omitted due to the short-time residence of liquid milk in the mouth. The simulated gastric fluid (SGF), simulated small intestinal fluid (SIF), and digestive enzyme solutions were prepared as described previously by Ménard et al. (2018) for an *in vitro* infant digestion model. The protocol used for the digestion without digestive enzymes was the same as described below, with SGF or SIF replacing the volume of the enzyme-containing solutions. In addition, blanks were prepared replacing ruminant milk samples with water prior to digestion. All digestions were carried out for each of the three independent milk samples of each species.

In vitro digestion without digestive enzymes was used to estimate the activity of milk proteases and the extent of digestion of milk proteins by milk proteases. On the other hand, the estimates obtained from digestion with gastric or pancreatic enzymes allowed for determining their influence on the activity of plasmin and cathepsin D in milk and protein hydrolysis. Together, these estimates were used to establish the contribution of milk proteases to protein digestion.

2.4.1. Gastric digestion

Briefly, 5 mL of milk samples were mixed with 3.5 mL of SGF (pH 5.3), 2.5 μL of 0.3 M $CaCl_2$, 0.25 mL of pepsin (268 U/mL; P7012, Sigma, USA), 0.25 mL of lipase (19 U/mL; P534781, Sigma, USA), 0.19 mL of 1 M HCl and water to a final volume of 10 mL. The solution was incubated at 37 °C in a water bath under shaking (80 rpm) for 60 min at pH 5.3. Gastric digestion was stopped by raising the pH to 7 with 1 M NaOH, and samples were collected and stored at −80 °C for analysis of protease activities and degree of protein hydrolysis within two weeks.

2.4.2. Small intestinal digestion

The remaining gastric medium (5 mL) was mixed with 2.13 mL of SIF, 0.63 mL of bile salts stock (3.1 mmol/L; B8631, Sigma, USA), 10 μL of 0.3 M $CaCl_2$, 1.25 mL of pancreatin solution (90 U/mL; P7545, Sigma, USA), 0.1 mL of 1 M HCl and water to a final volume of 10 mL. The solution was incubated at 37 °C in a shaking water bath (80 rpm) for 60 min at pH 6.6. Digestion was stopped by freezing sample aliquots at −80°C immediately after intestinal digestion to analyze protease activities and degree of protein hydrolysis within two weeks. Small intestinal digested samples refer to the samples collected after 60 min gastric

digestion plus 60 min intestinal digestion.

2.5. Determination of the degree of protein hydrolysis

The degree of protein hydrolysis was determined by measuring the free amino group (NH_2) released before and after *in vitro* digestion of the milk samples using the *o*-phthaldialdehyde (OPA) method (Church et al., 1983). Briefly, the OPA assays were carried out by adding 100 μL of milk sample to 1 mL of OPA solution (1.25 mL of 20% sodium dodecyl sulphate; 40 mg of OPA dissolved in 1 mL methanol; 100 μL of 2-mercaptoethanol, adjusted to a final volume of 50 mL with 0.1 M sodium tetraborate). The absorbance was measured after exactly 2 min at 340 nm in a UV/Visible spectrophotometer (Genesys, Thermo Fisher Scientific, USA). A calibration curve was prepared using standard glycine solutions (0 to 300 μg/mL). To determine the degree of protein hydrolysis, the total free NH_2 content of each ruminant milk sample was also needed to be determined. Briefly, samples were hydrolyzed first with 6 M HCl at 110°C for 24 h to cleave peptide bonds (Davies & Thomas, 1973), the acid was removed, and the total NH_2 was measured using the OPA method. The degree of protein hydrolysis was then calculated as follows:

$$\text{Degree of protein hydrolysis, } \% = \frac{\left(NH_{2(\text{digestion medium})} - NH_{2(\text{Blank})}\right)}{\left(NH_{2(\text{total})} - NH_{2(\text{Blank})}\right)} \times 100$$

where $NH_{2(\text{digestion medium})}$ is the concentration (μg/mL) of free NH_2 after gastric or small intestinal digestion with and without digestive enzymes, $NH_{2(\text{total})}$ is the total free NH_2 in each sample after acid hydrolysis, and $NH_{2(\text{blank})}$ is the free NH_2 in milk before digestion. Thus, only the new peptide bonds cleaved by milk and/or digestive proteases were considered in the calculation.

2.6. Determination of milk protease activities

Commercial fluorometric kits were used to measure the activities of plasmin (catalog K381-100, BioVision, California, USA), cathepsin D (Catalog K143-100, BioVision, California, USA), and elastase (Catalog K383-100, BioVision, California, USA), following the manufacturer's instructions. Briefly, 50 μL of diluted (1:4; milk:water) ruminant milk samples from different treatments (raw, heat treatment, and spray- and freeze-drying) and digestion steps (gastric and small intestinal) were mixed with 50 μL of substrate solution (plasmin substrate: D-Val-Leu-Lys-|-7-amino-4-methylcoumarin; cathepsin D substrate: 7-methylcoumarin-4yl-acetyl-Gly-Lys-Pro-lle-Leu-Phe-|-Phe-Arg-Leu-Lys-dinitrophenol-Arg-NH_2; elastase substrate: *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-|-7-amino-4-trifluoromethylcoumarin) in a 96-well black microplate. The microplate was incubated at 37 °C for 2 h, and the fluorescence was measured at 360 nm excitation and 450 nm emission filter for plasmin, 328 nm excitation and 460 nm emission filter for cathepsin D, and 380 nm excitation and 500 nm emission filter for elastase. Unfortunately, the manufacturer did not provide the cathepsin D standard. Instead, an active cathepsin D (catalog 9229, BioVision, California, USA) was bought to prepare the calibration curve (0–43 ng/μL). One unit of enzyme activity is the amount of enzyme that releases 1.0 μmol of substrate per min at pH 4 for cathepsin D and at pH 8 for plasmin and elastase at 37°C. Freeze-dried raw and heated milk samples were not used to compare protease activities after digestion with or without gastric (pepsin and lipase) or small intestinal (pancreatin) enzymes because freeze-drying was the process that less affected protease activities in all ruminant milk samples. Moreover, elastase activity was not determined in spray-dried milk samples digested with or without gastric and small intestinal enzymes due to its low concentration on caprine milk.

2.7. Peptides characterization via LC-MS/MS

2.7.1. Peptides extraction

Unwanted intact proteins were removed from the samples by mixing methanol at a 5:1 ratio to samples, and the resulting precipitated proteins were pelleted in a benchtop centrifuge at 17,000 × *g* for 30 min at 4 °C. Next, the supernatants were transferred to fresh tubes and concentrated to 40 μL from an original volume of 200 μL by vacuum evaporation in a Savant™ SpeedVac™ (model SC210A-230, Thermo Fisher Scientific, USA). Finally, the samples were centrifuged in a benchtop centrifuge at 17,000 × *g* for 20 min at 4°C prior to mass spectrometry analysis to remove any particulates.

2.7.2. Peptide identification

The peptides in the samples were separated by high-performance liquid chromatography (HPLC) on a DionexUltiMate™ 3000 RSLCnano system (Thermo Fisher Scientific, USA) with an online reversed-phase peptide trap (PepMap100 C18, 3 μm particle size, 75 μm ID, 2 cm length) and a reverse-phase capillary analytical column (PepMap C18, 2 μm particle size, 75 μm ID, 50 cm length; Thermo Fisher Scientific, USA). The HPLC system was coupled to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (MS) equipped with a higher-energy collision-induced dissociation (HCD) collision cell, an Orbitrap™ mass analyser and a Nanospray Flex™ Ion Source (Thermo Fisher Scientific, USA). The gradient was from 3 to 35% acetonitrile in 0.1% formic acid water in 60 min at a flow rate of 300 nL/min. The eluted peptides were analyzed by a data-dependent tandem MS acquisition (Top 10) method where a survey scan was performed at a resolution of 70,000 in the mass range of 375–1,600 *m/z,* and the top 10 most intense peptide ions were fragmented at a resolution of 17,500 to obtain their sequence information. The process was repeated every scan cycle in the chromatographic run with additional settings that enabled the MS to ignore repeating ions within specified time windows (15 s) and focus on peak apexes (8 s), allowing more low abundance ions to be targeted and improve overall coverage. The ionization source voltage was 1.5 kV with a capillary temperature of 250°C, an S-Lens RF level of 50%, an isolation width of 1.4 *m/z* and a normalized collision energy (NCE) of 27. The raw data files were searched against a combined database (NCBI, 12 Aug 2021, *Bos* taurus + *Capra* hircus + *Ovis* aries) using the Proteome Discoverer™ search engine (version 2.4.1.15, Thermo Fisher Scientific, USA) to obtain a list of peptides generated during digestion. The search parameters matched the instrument's specifications and included variables resulting from chemical treatment (carbamidomethyl of cysteine) and suspected natural modifications of proteins (oxidation of methionine, protein *N*-terminal acetylation). All protein hits were filtered to satisfy a false discovery rate (FDR) of 1% or better and have at least two unique peptides.

2.8. Enzyme prediction

The EnzymePredictor tool (https://bioware.ucd.ie/~enzpred/Enzpred.php) (Vijayakumar et al., 2012) was used to predict the milk endogenous and digestive enzymes involved in the hydrolysis of the peptide during the digestion. The identified peptides in non-dried and dried raw and heated (63°C/30 min or 75°C/15 s) ruminant milk samples were compiled by gastric and small intestinal digestion with digestive enzymes for each species.

2.9. Statistical analysis

Statistical analyses were performed using the Mixed Model procedure of SAS (SAS/STAT version 9.4; SAS Institute Inc., Cary, NC, USA). The initial model included a three-way ANOVA model to test the effect of heating (raw, 63°C/30 min, and 75°C/15 s), drying (non-dried, spray-drying, and freeze-drying), and species (bovine, ovine, and caprine) on the activity of proteases and degree of protein hydrolysis of milk samples

before and after *in vitro* digestion with or without digestive enzymes. The final model was chosen after removing interactions that did not influence the response variable using the log-likelihood ratio test.

The model diagnostics (e.g., normality, homogeneity of variances) for each response variable were tested after combining the Output Delivery System Graphics procedure and the Repeated statement of SAS before comparing the means. The repeated statement in the Mixed Model procedure was used to test the homogeneity of variances by fitting models with the Restricted Maximum Likelihood method and comparing them using the log-likelihood ratio test. Each response variable in the selected model had adjusted equal Studentized variances across treatments. Selected means were compared using the adjusted Tukey test when the F-value of the selected final ANOVA was significant ($p<0.05$). A trend was declared when $p<0.10$ but >0.05.

3. Results and discussion

To our knowledge, this study is the first to report the protease activities of processed ruminant milk under simulated gastric and small intestinal conditions. In agreement with the stated hypothesis, heating and drying processes affected plasmin, cathepsin D, and elastase activities of ruminant milk samples, but there were species-specific effects. Similarly, species-specific effects were observed for the plasmin activity of heated and spray-dried milk type after simulated gastric and small intestinal digestion with pepsin, lipase, and pancreatin. As postulated, milk proteases had an important contribution during simulated digestion with these enzymes.

3.1. Protease activities in raw ruminant milk

Among the non-dried raw samples, there were significantly higher activities ($p<0.05$) of plasmin (2.8- and 3.4-fold; Fig. 1A) and cathepsin D (1.4- and 1.7-fold; Fig. 2B and 2C) in caprine and ovine milk compared with bovine milk counterpart. These results are in accordance with Sharma et al. (2023), which also reported higher activities of plasmin (1.7-fold) and cathepsin D (1.4-fold) in caprine milk compared to bovine milk. Non-dried raw bovine (Fig. 2D) and ovine (Fig. 2E) milk had 38- and 40-fold higher activities of elastase than non-dried raw caprine milk (Fig. 2F), respectively.

The differences in enzyme activities between species could be partly due to the presence of different components of the proteolytic system (e.g., active enzymes, zymogens, and activators) across species (Leite et al., 2021). For instance, caprine milk has a higher level of plasminogen activator and a lower level of inhibitor compared to bovine milk, which may have contributed to converting plasminogen to its active enzyme, thereby increasing plasmin activity (Farrell et al., 2004). Moreover, other factors such as SCC and stage of lactation also can affect the enzyme activities (Farrell et al., 2004; Gautam et al., 2023; Politis et al., 1989). For example, caprine milk has higher SCC compared to bovine milk mastitis-free (Podhorecká et al., 2021). High SCC has been linked to high plasmin activity (Politis et al., 1989), which could explain the high plasmin activity observed in this study for raw caprine milk compared to raw bovine milk. Furthermore, Gautam et al. (2023) reported a significant increase in plasmin activity in caprine milk from early to mid-lactation. Conversely, no similar trend was observed in bovine milk, irrespective of the breed, suggesting that the modulation of plasmin activity during lactation differs between species.

3.2. Protease activities in ruminant milk after heat treatments and drying processes

When the processing was applied to the ruminant's milk, there was a significant effect ($p<0.001$) for the double interaction between species and heating process, and species and drying process for plasmin activity, and a triple interaction ($p<0.01$) between species, heating process, and drying process for cathepsin D and elastase activities, of milk samples (Supplementary Table 2).

Plasmin activity in caprine and ovine milk heated at 75°C/15 s (Fig. 1A) decreased ($p<0.05$) by 63 and 70%, respectively, compared with their raw counterparts, whereas plasmin activity did not change for heated bovine milk. In addition, plasmin activity in ovine milk samples heated at 63°C/30 min was reduced by 18% ($p<0.05$) compared to raw ovine milk. Heating did not affect cathepsin D and elastase activities in non-dried ruminant milk samples. These results contrast with those reported in our previous study (Leite et al., 2021), in which the heat treatment at 63°C/30 min increased plasmin activity by 45 and 100% in ovine whey and bovine casein fractions, respectively, and those reported by Hayes et al. (2001), where a decrease by 92% on the cathepsin D activity was observed in bovine milk after the heat treatment at 72.5°C for 15 s. The differences in methodological approaches may be the main cause of these contrasting results. For example, in our previous study, milk samples were ultracentrifuged (100,000 × *g* for 1 h at 20°C) and casein micelles were suspended in pH 8 to prepare the casein fraction, which may have destabilized the proteolytic system and contributed to

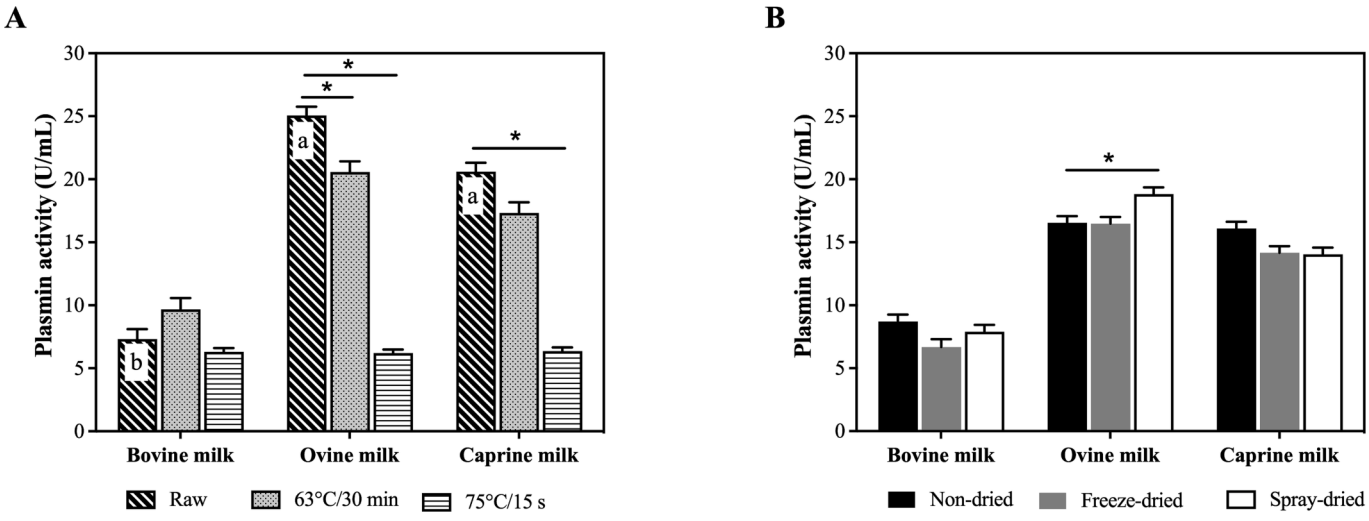

Fig. 1. Plasmin activity in ruminants' milk (bovine, ovine, and caprine) raw or heat treated (63°C/30 min and 75°C/15 s) (A) and subsequently kept non-dried or dried (freeze- and spray-dried) (B). There was a significant effect ($p<0.001$) for the interactions (species by heat (A) and species by drying (B)) (Supplementary Table 2). Values are means ± SEs, n = 9. Bars with different letters within raw milk (A) differ significantly ($p<0.001$). Asterisk represents significant differences ($p<0.05$) between raw and heat treated milk (A) and non-dried and dried milk within each species (B).

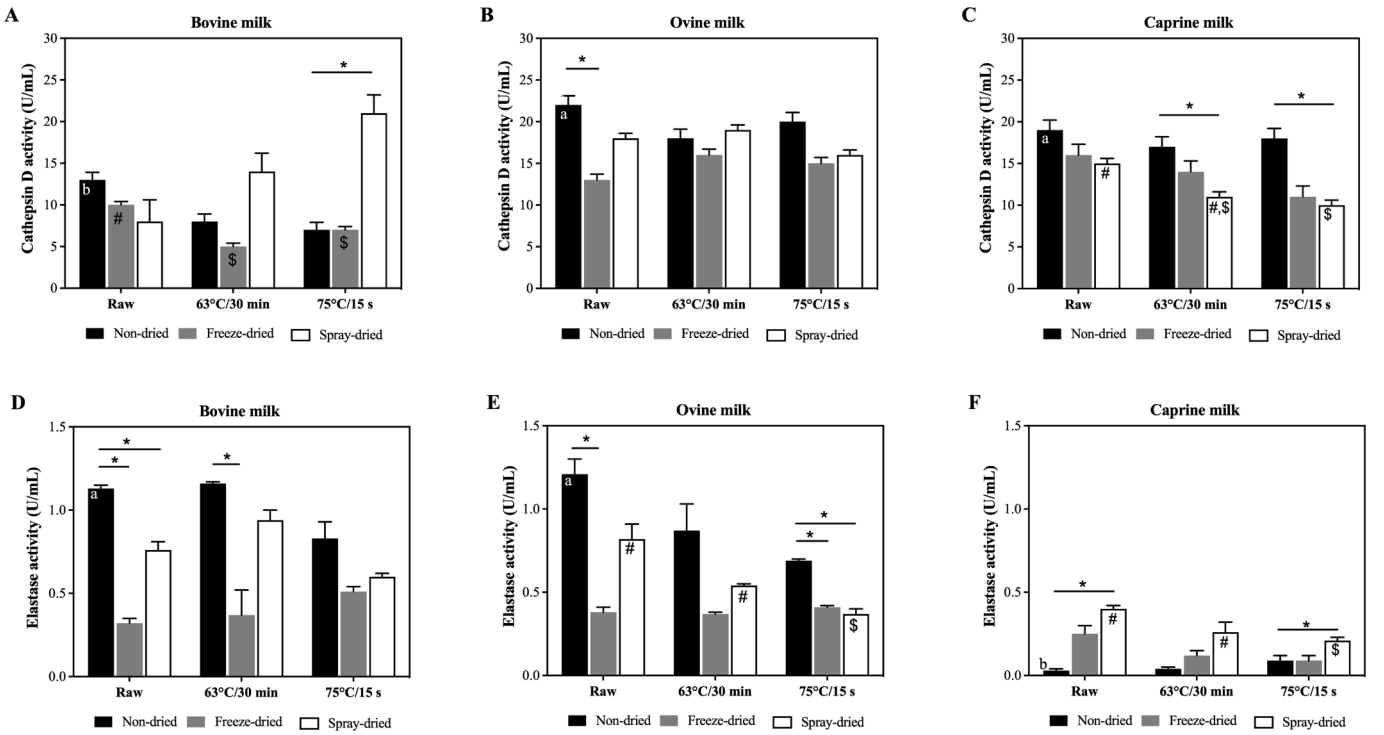

Fig. 2. Cathepsin D (A, B, and C) and elastase (D, E, and F) activities in bovine (A and D), ovine (B and E), and caprine (C and F) milk raw or heat treated (63°C/30 min and 75°C/15 s) and subsequently non-dried or dried (freeze- and spray-dried). There was a significant effect ($p<0.001$) for the interaction between species, heat and drying for each enzyme (Supplementary Table 2). Values are means ± SEs, n = 3. Bars with different letters across species within raw non-dried milk for each enzyme differ significantly ($p<0.001$). Bars with different symbols (# and $) in the spray- or freeze-dried samples across heat treatment (raw, 63°C/30 min, and 75°C/15 s) within species differ significantly ($p<0.001$). Asterisk represents significant differences ($p<0.05$) between non-dried and dried samples within each treatment.

plasmin activation. Moreover, Hayes et al. (2001) heated milk samples at 70°C for 10 min to inactive cathepsin D and then added cathepsin D stock solution into milk samples prior to applying the heat treatments. In contrast, our current study was designed to investigate the inactivation of indigenous enzymes in raw or processed skimmed milk, which may contain a higher fraction of the stable complex system than the purified cathepsin D stock solution used by Hayes et al. (2001), ensuring more stability to cathepsin D.

The mild heat treatment (63°C/30 min) preserved 57% more plasmin activity than the higher heat treatment (75°C/15 s). This observation could be explained by several factors, such as more intense heat treatment leading to protein aggregation (thiol-disulfide interactions) (Denis et al., 2001; Prado et al., 2006) and/or protein denaturation leading to structural modifications of milk proteases. For example, β-lactoglobulin is a heat-sensitive whey protein that undergoes conformational changes at temperatures above 70°C (Qian et al., 2017). These changes expose the sulfhydryl groups of cysteine residues, which are normally inside the protein structure, making them available for interaction with plasmin. This interaction between β-lactoglobulin and plasmin via thiol-disulfide interchange can lead to plasmin inactivation (Denis et al., 2001). In comparison, the decrease in plasmin activity was more significant in ovine milk, potentially due to its higher concentration of β-lactoglobulin than in bovine and caprine milk. Furthermore, heat can induce a higher level of denaturation in the whey protein of caprine milk than in bovine milk (Qian et al., 2017). Further work to determine the relationship between the degree of whey protein denaturation and protease activity is warranted.

There was no effect of spray- or freeze-drying on the activity of plasmin in bovine and caprine milk samples (Fig. 1B). In contrast, plasmin activity increased by 12% ($p<0.05$) when ovine milk was spray-dried, regardless of thermal treatment, compared to their non-dried counterparts (Fig. 1B).

The spray-drying process increased by 3-fold ($p<0.05$) the cathepsin D activity in bovine milk compared to their non-dried counterparts (heated at 75°C/15 s) (Fig. 2A). In contrast, this process decreased by 1.5- and 1.8-fold the cathepsin D activity of caprine milk heated at 63°C/30 min and 75°C/15 s, respectively, compared to non-dried caprine milk (Fig. 2C). Spray-drying did not affect the activity of cathepsin D in any ovine samples (Fig. 2B).

Across spray-dried samples, the elastase activity in ovine and caprine milk heat treated at 75°C/15 s decreased by 54 and 47%, respectively, compared to raw spray-dried milk counterparts (Fig. 2E and 2F). Conversely, elastase activity in raw and heated ovine milk samples at 75°C/15 s compared to caprine milk samples increased by 13- and 2.3-fold after spray-drying (Fig. 2F).

The destabilization of the proteolytic system, coupled with inhibitor inactivation caused by heating, as well as the waiting time at temperature of 4 and 20°C for the milk to undergo spray-drying, could have potentially contributed to the activation of plasmin in ovine milk (Anema, 2019; Leite et al., 2021), which could also explain the higher cathepsin D activity in spray-dried bovine milk heated at 75°C/15 s and higher elastase activity in spray-dried caprine milk heated at 75°C/15 s.

The presence of elastase in milk is mainly associated with polymorphonuclear neutrophils (PMNs), which undergo degranulation to release enzymes. During degranulation, specific granules within the PMNs fuse with the cell membrane, resulting in the extracellular release of their contents, which include elastase. It is known that heat affect the degranulation process of PMNs and subsequently impact the release of elastase in milk (Li et al., 2015). Based on this knowledge, it could be hypothesized that elastase was released from PMNs during the spray-drying, resulting in increased elastase activity in spray-dried compared to non-dried raw caprine milk. This phenomenal is particularly evident in caprine milk, possibly due to its higher concentration of PMNs compared to bovine and ovine milk (Kaskous et al., 2023).

However, further studies are required to confirm this hypothesis.

Freeze-drying did not influence ($p>0.05$) the cathepsin D activity of bovine and caprine milk samples kept raw or heated (63°C/30 min and 75°C/15 s) (Fig. 2A and 2C), and heated ovine milk samples (Fig. 2B). However, cathepsin D activity decreased ($p<0.05$) when raw ovine milk was freeze-dried (Fig. 2B).

The elastase activity in freeze-dried bovine milk samples decreased ($p<0.05$) by 71% in raw milk and 68% in heated milk at 63°C/30 min, but not at 75°C/15 s, compared to their non-dried counterparts (Fig. 2D). Both heating and drying processes affected the elastase activity in ovine and caprine milk compared to their raw or non-dried counterparts (Fig. 2E and 2F). For instance, freeze-drying reduced by 68 and 40% the activity of elastase in ovine milk samples kept raw or heated at 75°C/15 s, respectively, compared to non-dried counterparts (Fig. 2E).

Ice crystal formation and subsequent structure disruption of proteases and its zymogen during freeze-drying (Alinovi et al., 2020) were most likely why elastase activity in freeze-dried raw or heated bovine and ovine milk samples and cathepsin D in freeze-dried raw ovine milk were lower compared to their respective non-freeze-dried milk counterparts.

The protease activities observed in the study for the raw and processed milk samples were conducted in samples stored at −20 (dried samples) or −80°C (liquid samples) immediately after processing to avoid proteolysis during the storage. A previous study has shown that plasmin activity in processed milk (75°C for 15 s) increased during storage at 4°C for 96 h (Lu et al., 2009). Thus, further investigation is warranted to determine protease activities within the shelf-life of the raw and processed ruminant's milk used here.

3.3. Plasmin and cathepsin D activities in spray-dried milk after in vitro digestion

As mentioned above, spray-drying was the process that most affected the protease activities in all ruminant milk samples. Thus, spray-dried raw and heated milk samples were used to compare plasmin and cathepsin D activities before and after digestion with or without gastric enzymes (pepsin and lipase) or small intestinal enzymes (pancreatin). On the other hand, the elastase activity was not determined in any milk samples, as its concentration in caprine milk was low.

There was a significant interaction ($p<0.01$) between species, heating process, and digestion stage for plasmin and cathepsin D activities after digestion with or without digestive enzymes (Supplementary Table 3).

3.3.1. Plasmin activity after in vitro digestion without digestive enzymes

Plasmin activity in raw bovine samples was similar ($p>0.05$) before and after gastric digestion without gastric enzymes but higher ($p<0.05$) after small intestinal digestion without pancreatin (Fig. 3A). Plasmin activity of bovine milk in all heated conditions (vs pre-digestion levels) increased ($p<0.05$) after gastric (1.3-fold) or small intestinal (1.8-fold) digestion without their respective digestive enzymes, with the small intestinal digestion yielding significantly higher ($p<0.05$) activity level compared to gastric digestion (Fig. 3A).

The heat treatment of 75°C/15 s was the only condition that reduced (13–49%; $p<0.05$) plasmin activity in caprine and ovine milk samples

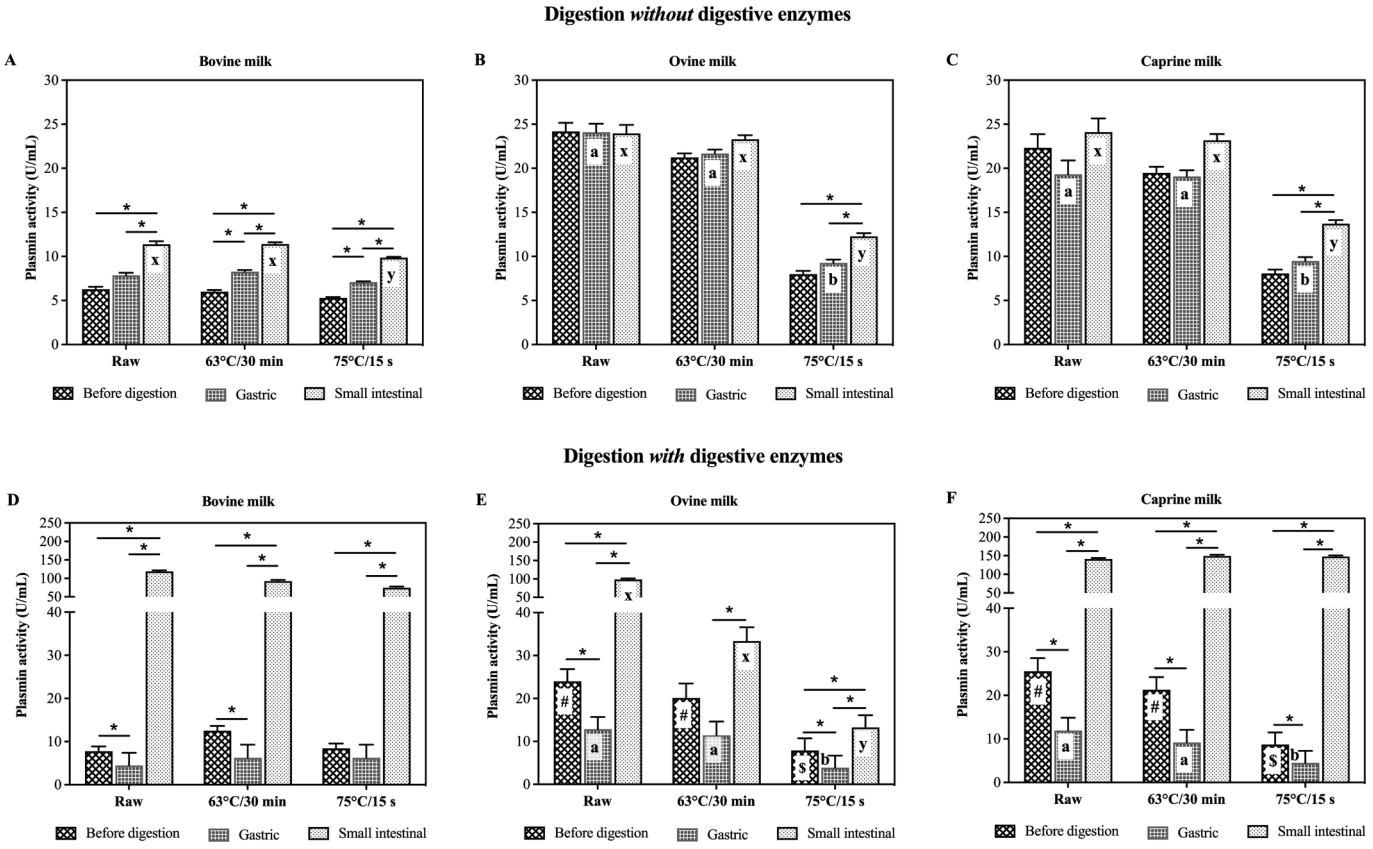

Fig. 3. Plasmin activity in bovine (A and D), ovine (B and E), and caprine (C and F) raw or heat treated (63°C/30 min and 75°C/15 s) spray-dried milk before and after simulated *in vitro* infant gastric (60 min) and small intestinal (60 min gastric + 60 min intestinal) digestion without (A, B and C) and with (D, E and F) digestive enzymes (pepsin, lipase, and pancreatin). There was a significant effect ($p<0.001$) for the interaction between species, heat treatment, and digestion at digestion without and with digestive enzymes (Supplementary Table 3). Values are means ± SEs, n = 3. Bars with different symbols or letters across heat treatments before digestion (# and $), gastric (a and b), and small intestinal (x and y) digestion differ significantly ($p<0.001$). Asterisk represents significant differences ($p<0.05$) between digestions within each heat treatment.

after gastric digestion without gastric enzymes, and bovine, ovine and caprine milk after small intestinal digestion without pancreatin, relative to raw milk or milk heated at 63°C/30 min (Fig. 3A, 3B and 3C).

No significant difference ($p>0.05$) was observed for the plasmin activity of ovine, and caprine milk samples were kept raw or heated at 63°C/30 min after gastric or small intestinal digestion without digestive enzymes (Fig. 3B and 3C). Despite this, the plasmin activity increased ($p<0.05$) in ovine (Fig. 3B) and caprine (Fig. 3C) milk samples heated at 75°C/15 s after small intestinal digestion without pancreatin, compared to pre-digestion and gastric digestion levels.

As previously mentioned, more intense heat treatment (75°C/15 s) may have caused structural changes in proteins, such as β-lactoglobulin, which could have enabled it to interact with plasmin, resulting in a decrease in plasmin activity. Although this heat treatment could have destabilized some components of the proteolytic system, it may not have impacted others, such as plasminogen. Therefore, based on the results obtained from the digestion without digestive enzymes, which reflect the influence of the pH on protease activity during digestion, it could be hypothesized that the transition from a simulated acidic condition in the stomach to higher pH (6.6) in the small intestine may have triggered plasminogen activation. This activation may have led to an increase in plasmin activity in the simulated small intestinal digestion, as optimal pH for plasminogen activation at 37°C falls within the pH of the simulated small intestinal digestion.

3.3.2. *Plasmin activity after in vitro digestion with digestive enzymes*

Plasmin activity in raw bovine, ovine, and caprine samples was lower ($p<0.05$) after gastric digestion with gastric enzymes but higher after small intestinal digestion with pancreatin (Fig. 3D, 3E and 3F).

The effects of thermal treatments on plasmin activity in bovine and ovine milk samples were inconsistent after gastric digestion with gastric enzymes (Fig. 3D and 3E). It decreased by 50% in the bovine milk sample heated at 63°C/30 min but was unchanged in bovine milk heated at 75°C/15 s, compared to pre-digestion levels (Fig. 3D). Oppositive behavior was observed in ovine milk, in which the level of plasmin in milk heated at 63°C/30 min was not affected, and a significantly lower activity (51%) was found in milk heated at 75°C/15 s after gastric digestion with enzymes compared to pre-digestion levels (Fig. 3E). Plasmin activity in caprine milk samples in all heated conditions (vs pre-digestion levels) decreased ($p<0.05$) after gastric but increased after small intestinal digestion, both with their respective enzymes (Fig. 3F). As observed above for the digestion without pancreatin, for gastric digestion, plasmin activity in ovine and caprine milk samples heated at 75°C/15 s decreased by 70 and 62%, respectively, compared to raw gastric digested samples. Moreover, plasmin activity in digested ovine milk samples heated at 75°C/15 s was also reduced (87%) during the small intestinal digestion stage.

In general, after digestion with gastric enzymes, the plasmin activity decreased by 43 to 57% in most milk samples compared to pre-digestion levels. This decrease could be ascribed to the breakdown of plasmin by pepsin used in the gastric digestion stage. On the other hand, most milk samples had higher plasmin activity after the small intestinal digestion with small intestinal enzymes compared to pre-digestion levels or after digestion with gastric enzymes. These increases could be explained by

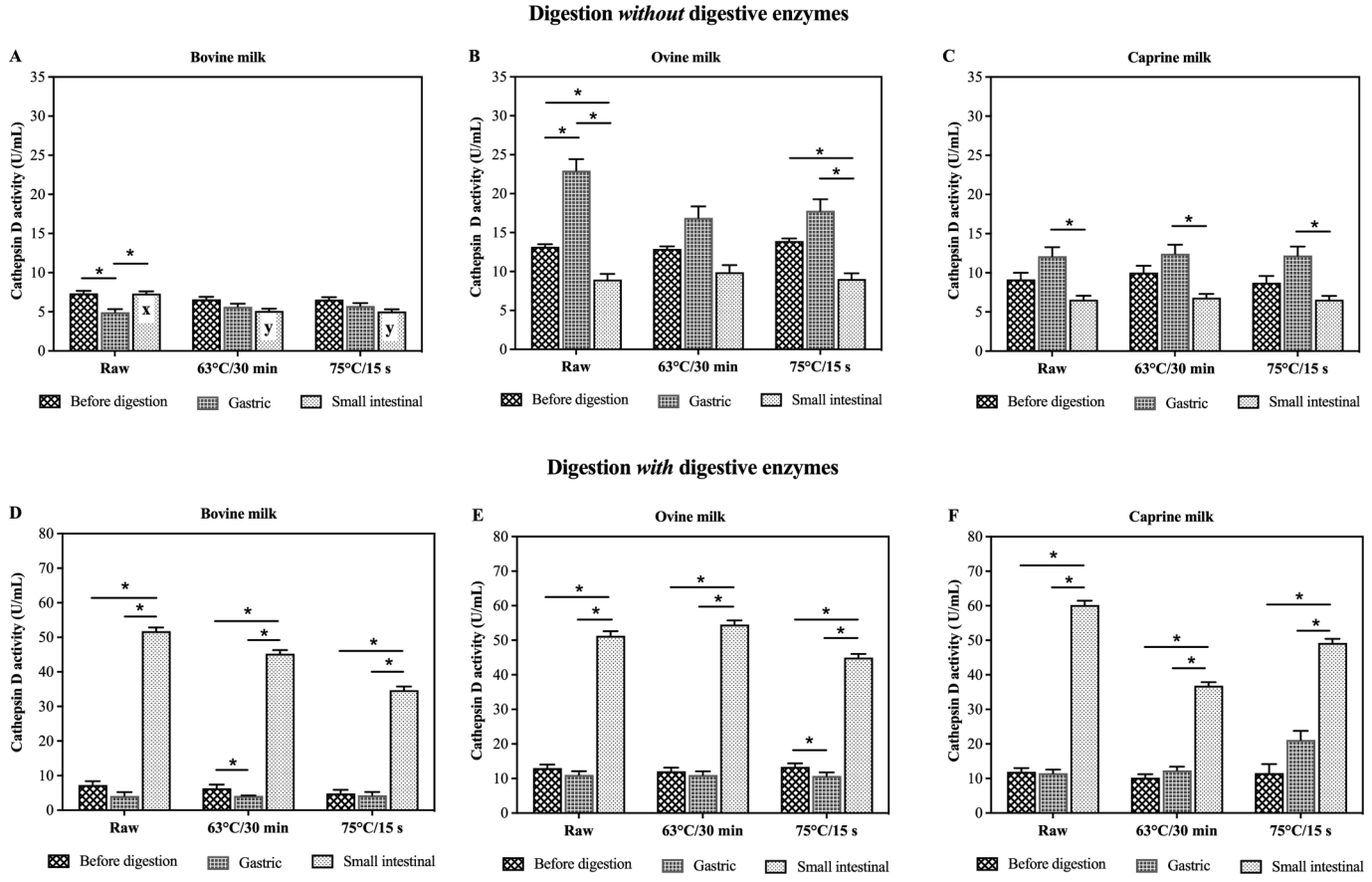

Fig. 4. Cathepsin D activity in bovine (A and D), ovine (B and E), and caprine (C and F) raw or heat treated (63°C/30 min and 75°C/15 s) spray-dried milk before and after simulated *in vitro* infant gastric (60 min) and small intestinal (60 min gastric + 60 min intestinal) digestion without (A, B and C) and with (D, E and F) digestive enzymes. There was a significant effect ($p<0.001$) for all main factors (species, heat treatment, and digestion) and the interactions between species and digestion at digestion without and with digestive enzymes (Supplementary Table 3). Values are means ± SEs, n = 3. Bars with different letters across heat treatments within small intestinal digestion (x and y) (A) differ significantly ($p<0.001$).

the activation of plasminogen to plasmin by pancreatic proteases, as reported for trypsin (Kocholat et al., 1952) and chymotrypsin (Deng et al., 2018), the change in pH as observed in the *in vitro* digestion without enzymes, or by the hydrolysis of plasmin substrate by the pancreatic enzymes, as observed in the blanks containing digestive enzymes alone.

3.3.3. Cathepsin D activity after in vitro digestion without digestive enzymes

The cathepsin D activity in all milk samples was more affected by the digestion stage without digestive enzymes than heat treatments (Fig. 4A, 4B and 4C). For instance, the cathepsin D activity decreased by 33% in raw bovine milk and increased by 43% in raw ovine milk samples (but not in caprine milk) after gastric digestion without gastric enzymes, compared to pre-digestion levels (Fig. 4A and 4B).

The finding in bovine milk could be explained by a lower concentration of procathepsin D and/or cathepsin D inactivation by other active proteases in bovine milk compared to ovine milk. Moreover, the increase in cathepsin D activity in ovine milk might be attributed to gastric acidity, which may have activated procathepsin D to cathepsin D, as reported elsewhere for human milk (Demers-Mathieu et al., 2018).

The cathepsin D activity in raw bovine milk was higher after small intestinal digestion than gastric digestion, both without digestive enzymes, but similar to pre-digestion levels (Fig. 4A). In contrast, cathepsin D activity in raw ovine milk samples was higher (74%) after gastric digestion but lower (33–61%) after small intestinal digestion compared to pre- and gastric digestion without small intestinal enzymes (Fig. 4B). A similar trend was observed in cathepsin D activity in ovine milk samples heated at 75°C/15 s, showing lower activity after small intestinal digestion compared to pre-digestion levels or levels obtained after gastric digestion (Fig. 4B).

The cathepsin D activity in caprine milk samples kept raw or heated (63°C/30 min and 75°C/15 s) was not affected after gastric digestion without gastric enzymes (Fig. 4C); however, lower activity ($p<0.05$) was found after small intestinal digestion compared to gastric digestion (Fig. 4C).

After the small intestinal digestion without intestinal enzymes, the cathepsin D activity of raw and/or heated bovine, ovine and caprine milk was similar or lower to the levels measured prior to digestion, likely due to increased pH from the simulated stomach (pH 5.3) to the simulated small intestine (pH 6.6) conditions or cathepsin degradation at neutral pH in the simulated small intestinal conditions, both decreasing the cathepsin D activity (Buck et al., 1992). Holton et al. (2014) reported that cathepsin D has an optimal pH of 3.5 but still retains over 25% activity at pH 5, which suggests that the conditions used in this study to simulate the small intestine may not be optimal for the activation of cathepsin D.

3.3.4. Cathepsin D activity after in vitro digestion with digestive enzymes

When digestive enzymes were used, the cathepsin D activity of all milk species was also affected ($p<0.05$), mainly by the digestion stage (Fig. 4D, 4E and 4F). However, there was no difference in cathepsin D activity in all milk samples kept raw or heated at 63°C/30 min after gastric digestion with gastric enzymes, compared to pre-digestion levels, except for bovine milk heated at 63 °C/30 min and ovine milk heated at 75°C/15 s samples, where a decrease of activity was noted (Fig. 4D and 4E). Otherwise, the cathepsin D activity in all milk samples increased by 3- to 12-fold after small intestinal digestion with digestive enzymes, compared to pre-digestion levels or levels after gastric digestion with gastric enzymes.

The stability of cathepsin D activity under the gastric condition in most milk samples could be due to the inhibitor-binding property of pepsin, limiting the activation of procathepsin D to cathepsin (Conner, 1989). Moreover, the increase in cathepsin D activity under small intestinal conditions is likely due to the hydrolysis of cathepsin substrates (added to milk samples for activity measurement) by pancreatic proteases rather than the neutral pH in the small intestinal lumen, activating milk procathepsin D to cathepsin D and increasing cathepsin activity (Demers-Mathieu et al., 2018).

3.4. Degree of protein hydrolysis of milk after in vitro digestion

The effects of gastric and small intestinal digestion on the degree of protein hydrolysis in milk samples were determined using an *in vitro* model of infant digestion with and without pepsin (gastric), lipase (gastric), and pancreatin (small intestine). The pre-digestion amount of free NH_2 groups was subtracted from gastric and small intestinal digestion to calculate the free NH_2 resulting from gastric or small intestinal digestion.

The heating and drying processes did not influence ($p>0.05$) the degree of protein hydrolysis of any milk samples after gastric digestion without (Fig. 5) and with (Fig. 6) digestive enzymes (Supplementary Table 4), either as individual factors or in the different interactions. However, in small intestinal digestion, there was a significant effect for the species ($p<0.001$) and drying process ($p<0.05$) in digestion without small intestinal enzymes and a triple interaction between species, heating, and drying processes ($p<0.01$) at digestion with small intestinal enzymes (Supplementary Table 4).

The caprine milk samples had the highest degree of protein hydrolysis compared to bovine or ovine milk samples, with ovine milk having the lowest hydrolysis levels ($p<0.05$, Fig. 5) before digestion or after gastric digestion without or with gastric enzymes (Figs. 5 and 6A). In addition, there was a higher degree of protein hydrolysis in caprine milk samples than bovine or ovine milk samples after small intestinal digestion without pancreatin, but similar levels in bovine and ovine milk samples.

Differences were also observed in the degree of protein hydrolysis between digestion with and without digestive enzymes during *in vitro* gastric digestion. For example, ovine, caprine, and bovine milk showed 2.4, 1.4 and 1.3-folds, respectively, more digested proteins without than with the presence of digestive enzymes. This result suggests that pepsin hydrolyzed some of the milk proteases leading to a fewer cleaved peptides bond.

The highest degree of protein hydrolysis in caprine milk samples could be due to the higher activity of plasmin and cathepsin D, as observed in this study, differences in protein composition, which have different susceptibility to proteases (Dupont & Tomé, 2020), and more unfolded milk proteins based on the greater number of free NH_2 prior to digestion. For instance, caprine milk (63 g/100 g protein) has a higher concentration of β-casein compared to bovine (42 g/100 g protein) milk and ovine (30 g/100 g protein) milk, which is the protein preferentially cleaved by plasmin (Deglaire et al., 2019; Khan et al., 2019). Additionally, caprine milk produces a fragile curd that retains a high proportion of water compared to milk from other species, which contributes to the access of proteases to milk proteins. Furthermore, the higher degree of protein hydrolysis in mainly caprine milk after small intestinal digestion compared to gastric digestion without digestive enzymes could be explained by the activation of plasmin or by pH effects on protease activity in caprine milk, as discussed above, which contributed to the increase the degree of protein hydrolysis.

Drying neither thermal process did not affect ($p>0.05$) the degree of protein hydrolysis in bovine milk samples after the small intestinal digestion with pancreatin (Fig. 6B). In contrast, with either freeze-drying or spray-drying (vs non-dried samples), the degree of protein hydrolysis in raw ovine milk samples increased by 2.1-fold (Fig. 6C), while for the raw caprine milk, the degree of hydrolysis decreased by 15% ($p<0.05$) (Fig. 6D) after small intestinal digestion with pancreatin. Furthermore, there was no effect of thermal treatment either at 63°C/30 min or 75°C/15 s on the degree of protein hydrolysis in caprine milk samples, except a lower degree in spray-dried caprine milk samples compared to freeze-dried samples, both heated at 75°C/15 s (Fig. 6D).

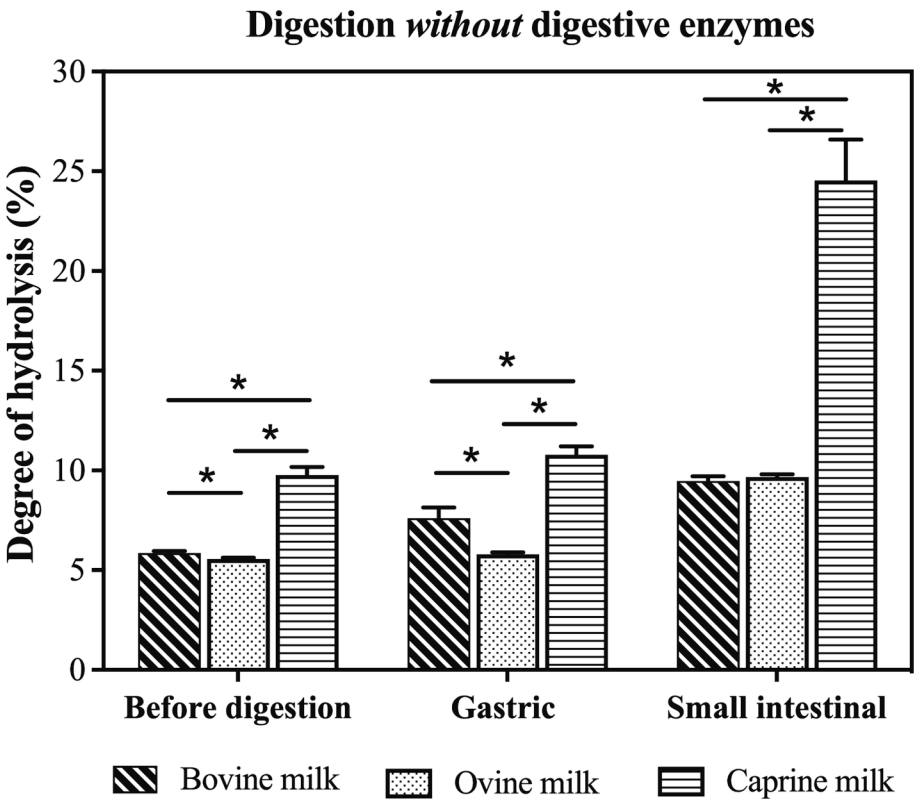

Fig. 5. Degree of protein hydrolysis in ruminant's milk (bovine, ovine, and caprine) before and after simulated *in vitro* infant gastric (60 min) and small intestinal (60 min gastric + 60 min intestinal) digestion without digestive enzymes, measured by the release of free amino groups. Digestion without digestive enzymes refers to milk digested without pepsin, lipase, and pancreatin but using the corresponding buffers and conditions (e.g., pH). Values are means ± SEMs, n = 27. There was a significant effect ($p<0.001$) for the species factor at before and gastric digestion and for the species and drying (non-, spray- and freeze-dried) factors at small intestinal digestion ($p<0.05$) (Supplementary Table 4). The heat treatment (raw, 63°C/30 min, and 75°C/15 s) did not influence ($p>0.05$) any of the digestions. At small intestinal digestion, the degree of hydrolysis was 14.9a, 14.7ab, and 14.1b for freeze-, spray-, and non-dried milk, respectively. To determine the degree of hydrolysis at gastric and small intestinal digestions, the free amino group before digestion were substrate as shown in Materials and Methods. Asterisk represents significant differences ($p<0.05$) between species within digestions.

3.5. Prediction of cleaved peptide bonds by milk proteases and small intestinal enzymes after in vitro digestion

In this study, plasmin was the protease predicted to cleave more peptides during gastric digestion with digestive enzymes (pepsin), followed by cathepsin D and elastase (Supplementary Fig. 2A). Milk proteases could explain 88–99% of the protein hydrolysis during gastric digestion. This result agrees with Demers-Mathieu et al. (2018) for human milk, which showed through the peptidomic analysis that milk proteins were mainly digested by plasmin and cathepsin D present in milk during gastric digestion. Dallas et al. (2015) reported that infants have a lower digestive capacity to hydrolyze proteins than adults due to the low concentration of gastric acid in the stomach. The relatively neutral pH value in the stomach of infants can affect the activity of gastric enzymes, such as pepsin, which works optimally at pH 3. Therefore, milk enzymes play a crucial role in compensating for the lower digestive function in infants (Dallas et al., 2015).

After the small intestinal digestion, a higher number of peptide bonds cleaved by digestive proteases was observed when compared with gastric digestion (Supplementary Fig. 2b). This result is explained by pancreatic proteases (trypsin and chymotrypsin), and by the potential activity of pepsin under the simulated infant small intestinal digestion (greater number of peptide bonds broken during small intestinal digestion vs gastric digestion). This greater number of peptide bonds identified for pepsin could be attributed to the unfolding structure of milk proteins by pancreatic proteases, which increases the pepsin's accessibility to its cleavage specificity amino acids, such as phenylalanine tryptophan and tyrosine at position P1 and P1′ (Castañeda-Valbuena et al., 2022). The peptidome results also confirmed that digestive enzymes played a greater role in the small intestinal digestion of milk proteins than milk proteases. Nevertheless, milk proteases contributed 25–40% of the degree of protein hydrolysis after small intestinal digestion (Supplementary Fig. 2b). It is important to highlight that the number of peptide bonds cleaved from the gastric to the small intestinal digestion needs to be carefully interpreted, as peptides identified in the gastric digestion would not have been identified in the small intestinal digestion if they had been further hydrolyzed. This limitation could explain why the number of peptide bonds cleaved by milk proteases observed after gastric digestion was higher than after small intestinal digestion. It has been shown in the porcine model that the digested protein entering the small intestine is one of the factors influencing the digestibility of amino acids throughout the small intestine (Montoya et al., 2018). Thus, our gastric results highlight the importance of milk proteases to the overall digestion and absorption of milk proteins, and further work to understand this effect *in vivo* is warranted.

Ovine milk shows more peptide bonds cleaved by digestive enzymes (pepsin and trypsin + chymotrypsin) compared to bovine and caprine milk at small intestine digestion (Supplementary Fig. 2b). This result might be due to ovine milk's higher concentration of total proteins (Supplementary Table 1) and β-lactoglobulin (Ruprichova et al., 2014), which is resistant to digestion by pepsin but susceptible to small intestinal proteases as reported in both *in vivo* and *in vitro* studies (Bouzerzour et al., 2012; Sanchón et al., 2018).

3.6. Strengths and limitations

To our knowledge, this study is the first to report protease activities of heat- and dry-processed ruminant milk samples under simulated gastric and small intestinal conditions. However, it is important to highlight that only static *in vitro* digestion models were used, which do not represent *in vivo* digestion. Thus, the *in vitro* results need to be carefully interpreted. Nevertheless, the results obtained here provide some mechanistic understanding of milk protease activation and/or inactivation under simulated conditions and their potential contribution to digestion.

4. Conclusion

This study showed that the main endogenous enzymes in ruminants' milk, plasmin, cathepsin D, and elastase, remained active after heating

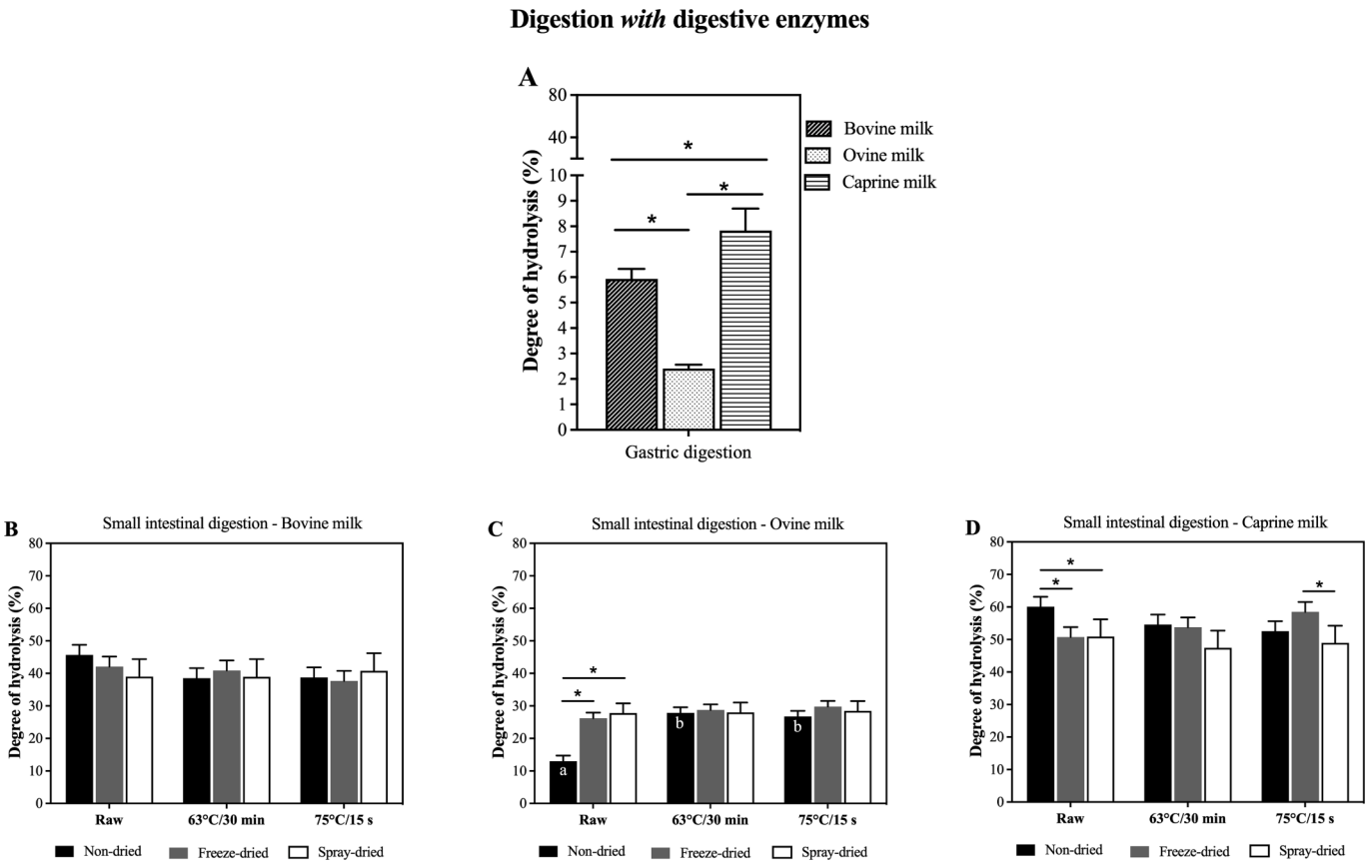

Fig. 6. Degree of protein hydrolysis in ruminant's milk (bovine, ovine, and caprine) after simulated *in vitro* infant gastric (60 min; A) and small intestinal (60 min gastric + 60 min intestinal; B, C, and D) digestion with digestive enzymes, measured by the release of free amino groups. Digestion with digestive enzymes refers to the digestion with pepsin and lipase in the gastric digestion stage, followed by pancreatic enzymes during small intestinal digestion. Values are means ± SEMs, n = 27 (A) and n = 3 (B, C, and D). There was a significant effect ($p<0.001$) for the species factor at gastric digestion (A) and the interaction between species, heat (raw, 63°C/30 min, and 75°C/15 s) and drying (non-, spray- and freeze-dried) processes in the small intestinal digestion stage (B, C, and D). Asterisk represents significant differences ($p<0.05$) between species within gastric digestion (A) and drying process (B, C and D) within heat treatments. Bars with different letters in non-dried samples within heat treatment (raw, 63°C/30 min, and 75°C/15 s) differ significantly ($p<0.001$).

and/or drying, but their activities varied according to the milk species. In addition, some differences in protease activities caused by mild heat treatment disappeared under simulated digestion with pepsin, lipase, and pancreatin. Endogenous milk enzymes play an important role in the hydrolysis of milk proteins during gastric *in vitro* digestion and a secondary role during *in vitro* small intestinal digestion. Further research is warranted to investigate the impact of processes on protease activities and its consequence on the hydrolysis of milk proteins in an *in vivo* study.

CRediT authorship contribution statement

Juliana A.S. Leite: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Carlos A. Montoya:** Conceptualization, Formal analysis, Methodology, Software, Supervision, Writing – review & editing. **Simon M. Loveday:** Methodology, Supervision, Writing – review & editing. **Jane A. Mullaney:** Formal analysis, Writing – review & editing. **Trevor S. Loo:** Data curation, Methodology, Software, Writing – review & editing. **Warren C. McNabb:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Nicole C. Roy:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank Riddet Institute and AgResearch for providing access to facilities.

Funding

This research was funded by the Ministry of Business, Innovation and Employment (MBIE) (Grant C10X1709) from New Zealand and the Riddet Institute.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136979.

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