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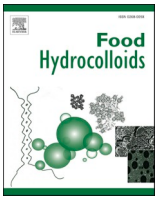

Decalcification strongly affects *in vitro* gastrointestinal digestion of bovine casein micelles under infant, adult and elderly conditions

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ABSTRACT

Effects of 0–90% decalcification on the coagulation behavior and gastrointestinal digestibility of bovine casein micelles were studied *in vitro* under infant, adult and elderly conditions. Gastric coagula became looser with increasing level of decalcification, especially up to 40% under the infant condition and up to 69% under the adult and elderly conditions. Casein degradation showed a similarly increasing trend with increasing level of decalcification under the three age conditions. Casein degradation was fastest under the adult condition, but slowest under the elderly and infant conditions at 0–40 and 69–90% decalcification, respectively. Formation of free amino groups and small peptides increased with increasing level of decalcification and in order of infant, elderly and adult conditions. In intestinal phase, the remaining caseins were very rapidly degraded. Formation of free amino groups and smaller peptides showed minor differences with increasing level of decalcification, but increased in order of infant, elderly and adult conditions. This study highlighted the different level of decalcification suitable for diverse age populations to improve the gastrointestinal digestibility of bovine casein micelles.

1. Introduction

Bovine casein micelles are supramolecular assemblies of α_{s1}-, α_{s2}-, β- and κ-caseins, with the former three caseins forming the internal structures of the micelles mainly through their hydrophobic regions and phosphoserine residues connected with colloidal calcium (Ca) phosphate (CCP). The κ-casein is predominantly located on the surface of the micelles and provides electrostatic and steric stabilization to the micelles through its hairy polyelectrolyte region. The micelles are in a dynamic equilibrium with a serum phase containing free caseins and soluble minerals such as Ca, phosphate and citrate (Dalgleish & Corredig, 2012). Casein micelles, comprising the major proteins in bovine milk, are important sources of amino acids and minerals. Casein micelles are generally considered as sources of slow dietary proteins, compared with whey proteins that are soluble even in the acidic stomach and can quickly enter the small intestine where degradation by pancreatin rapidly occurs (Roy et al., 2022). Casein micelles are prone to coagulation in the stomach due to the collapse of κ-casein hairy layers with decreasing pH, the removal of κ-casein hairy layers by pepsin and the occurrence of inter-micellar Ca bridging, which prolongs the gastric emptying and delays the delivery of caseins and their derived polypeptides to the small intestine (Huppertz & Chia, 2021).

Gastrointestinal behavior of casein micelles is associated with their amount of Ca. Zou et al. (2022) reported that during an infant *in vitro* gastric digestion, dense clusters were observed for bovine micelles, while no visible clusters were observed for human micelles, due partially to the lower amount of Ca in human micelles (Yang, Liu, & Zhou, 2022). Formation of dense gastric clusters is related to the occurrence of reflux esophagitis and constipation in infants (Nurko, Benninga, Solari, & Chumpitazi, 2022). Huppertz and Lambers (2020) reported that for bovine micelles in a model infant formula, 13–63% decalcification achieved using acidification followed by dialysis resulted in no visible clusters and faster casein degradation during an infant *in vitro* gastric digestion. Wang, Ye, Lin, Han, and Singh (2018) reported that during an adult *in vitro* gastric digestion, looser and more fragmented clusters and hence a faster casein degradation were observed for bovine micelles in

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milk protein concentrate with 42% decalcification using ion-exchange, compared to that without decalcification. Liao et al. (2022) also reported that decalcification of bovine casein micelles by adding sodium citrate resulted in weaker clusters and faster casein degradation during an adult *in vitro* gastric digestion, due to an increased accessibility of caseins to pepsin.

Gastrointestinal digestibility of casein micelles differs among diverse age populations with different gastrointestinal conditions such as the secretion levels of gastric acid and digestive proteases (Mackie, Mulet-Cabero, & Torcello-Gómez, 2020). Ménard et al. (2018) reported that *in vitro* gastric degradation of caseins in an infant formula was slower under infant condition than that under adult condition, due to the lower level of pepsin and higher pH under the infant condition. Dupont et al. (2010) also reported that *in vitro* degradation of β-casein was slower under infant condition compared with adult condition. Aalaei, Khakimov, De Gobba, and Ahrné (2021) reported that *in vitro* gastric degradation of caseins in ultra-high-temperature sterilized milk was slower under elderly condition than that under adult condition, due to the lower level of pepsin used under the elderly condition. Hernández-Olivas, Muñoz-Pina, Sánchez-García, Andrés, and Heredia (2020) also reported that proteolysis during *in vitro* digestion of milk, yogurt and cheese was slower under elderly condition than that under adult condition.

Previous researches have reported the *in vitro* digestibility of decalcified bovine casein micelles solely under infant or adult condition (Huppertz & Lambers, 2020; Wang et al., 2018), while lacking a systematic comparison among different age populations, in whom the digestion behaviors of bovine micelles might respond differently to their decalcification. Therefore, this study investigated the *in vitro* gastrointestinal digestibility of bovine micelles with a wide range of decalcification under infant, adult and elderly conditions, with the mechanism interpretation being explored by comparing the coagulation and proteolysis behaviors. This study aimed to better understand the age-dependent response in digestion behaviors of bovine micelles towards their decalcification, thus shedding light on the critical level of decalcification suitable for diverse age populations to improve the gastrointestinal digestibility.

2. Materials and methods

2.1. Materials

Fresh bovine milk from Holstein cows was purchased from Tianzi Dairy Co., Ltd. (Wuxi, Jiangsu, China). Amberlite SR1L Na ion-exchange resin was purchased from Rohm and Haas (Philadelphia, PA, USA). Porcine pepsin (3880 U/mg), porcine pancreatin (7.4 U trypsin/mg), porcine bile, pepstatin A, Pefabloc SC, hemoglobin, p-toluene-sulfonyl-L-arginine methyl ester (TAME), fluorescein isothiocyanate, *o*-phthaldialdehyde (OPA), cytochrome C (12,384 Da), bacitracin (1422 Da), Gly-Gly-Tyr-Arg (451 Da) and Gly-Gly-Gly (189 Da) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Fractionation and decalcification of bovine casein micelles

The raw milk was skimmed at 50 °C and 9600 rpm using a CLARA 20LFCO cream separator (Alfa Laval Corp. AB, Lund, Sweden), and the resulting skim milk (<1 g fat/L) was microfiltered at 50 °C using a GCM-C-03 unit (Guochu Technology Co., Ltd., Xiamen, Fujian, China) loaded with 3 tubular ceramic Isoflux™ membranes with a pore diameter of 1.4 μm (0.312 m^2 surface area; TAMI Industries, Nyons, France). Pasteurization of skim milk would induce denaturation and association of whey proteins with casein micelles, thus co-enriching the associated whey proteins in micelle powders as well as affecting the coagulation and proteolysis of micelles under gastric conditions, which would be avoided by using microfiltration instead (Ye et al., 2019). The permeate was collected and microfiltered at 50 °C using another 3 tubular ceramic membranes with a pore diameter of 0.1 μm (Guochu Technology). The microfiltration was done until a volume concentration factor of 3 was achieved, and the retentate was mixed with 2 vol of distilled water and then diafiltered under the same condition mentioned above. The diafiltration step was repeated 2 more times, and the resulting retentate was divided into 5 portions. One portion was kept as the untreated control, and the other 4 portions were mixed with Amberlite SR1L Na resin at ratios of 1.1, 3.8, 8.0 and 16.0 g resin/100 g retentate, respectively. The level of decalcification for casein micelles does not increase linearly with increasing addition of resin, and preliminary experiments involving different added amounts of resin showed that the used four amounts resulted in three levels of decalcification distributed roughly evenly between the non-decalcification and maximum decalcification. After being stirred at 500 rpm for 4 h using a C-MAG HS7 magnetic stirrer (IKA Werke GmbH & Co. KG, Staufen, Germany), the mixtures were filtered using 2-layered cloths. The filtrates were added dropwise with 2 M HCl under stirring to restore the pH to that of the control (6.9), and then lyophilized using a BenchTop Pro freeze drier (SP Scientific Co., Stone Ridge, NY, USA). The casein micelle powders contained ~85.9% of total proteins and ~94.8% of caseins relative to total proteins, as determined using the ISO (2001, 2004) methods.

2.3. Reconstitution of casein micelle powders

The casein micelle powders were reconstituted in ultrapure water to obtain dispersions with a casein content of 12 g/L. The dispersions were stirred at 50 °C and 400 rpm for 1 h using the C-MAG HS7 magnetic stirrer, and then homogenized at 30 MPa for 3 passes using an AMH-3 unit (ATS Engineering Ltd., Shanghai, China), followed by overnight storage at 4 °C to achieve full hydration. Preliminary experiments showed that optical absorbance of the filtrates collected in Section 2.2 remained almost unchanged after being homogenized at 30 MPa for 3 passes (Table S1), suggesting a limited effect of the applied homogenization on casein micelle structures for the obtained level of decalcification.

2.4. Determination of Ca distribution

The casein micelle dispersions were ultracentrifuged at 25 °C and 100,000 ×*g* for 1 h using an Optima L-100XP ultracentrifuge (Beckman Coulter, Inc., Indianapolis, IN, USA), and the resulting supernatants were ultrafiltered at 25 °C and 2500 ×*g* for 1 h using Vivaspin 6 concentrators with a molecular weight cut-off of 10 kDa (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The micelle dispersions and their ultrafiltrates were hydrolyzed in nitric-perchloric acid (4:1, v/v) using a MARS Microwave Digestion System (CEM Corp., Matthews, NC, USA), and then determined using an iCAP TQ inductively coupled plasma mass spectrometer (Thermo Fisher Scientific, Inc., Bremen, Germany) for total and permeable Ca, respectively. The difference between total and permeable Ca was assumed to be the amount of non-permeable Ca that was associated directly with caseins or integrated in CCP (Huppertz & Lambers, 2020). The difference in non-permeable Ca between non-decalcified and decalcified micelles, expressed as a percentage of the amount of non-permeable Ca in non-decalcified micelles, was assumed as the level of decalcification.

2.5. Determination of turbidity

Absorbance of the casein micelle dispersions was measured in a 1 cm path-length quartz cuvette using an UV-2700 spectrophotometer (Shimadzu Corp., Kyoto, Japan) at a wavelength of 860 nm (Xu et al., 2016). Relative turbidity was expressed as a ratio of absorbance of the decalcified micelle dispersion relative to that of the non-decalcified micelle dispersion.

2.6. In vitro gastrointestinal digestion

Three static *in vitro* gastrointestinal digestion conditions for infants, adults and elderly were used. Before performing digestion, the enzyme activities for porcine pepsin and pancreatin were measured using hemoglobin and TAME as the substrates, respectively, as described by Brodkorb et al. (2019). Digestion of the casein micelle dispersions was done in 50 mL jacketed glass beakers maintained at 37 °C using a MP-501A circulating water bath (Yiheng Technical Co., Ltd., Shanghai, China) and with continuous stirring at 220 rpm using a RO-10 Power magnetic stirrer (IKA Werke GmbH & Co. KG).

The infant *in vitro* gastrointestinal digestion was performed according to the method of Ménard et al. (2018) with slight modifications. This method was developed based on *in vivo* data derived from the gastrointestinal tract of full-term newborn aged ~1 month (Bourlieu et al., 2014). For gastric digestion, each casein micelle dispersion was mixed (63:37, v/v) with a simulated gastric fluid (SGF, 724.3 U pepsin/mL, pH 5.3, 13 mM KCl, 94 mM NaCl), followed by readjusting the pH to 5.3. After gastric digestion, the digesta were adjusted to pH 7.0 using dropwise addition of 2 M NaOH, and then mixed (62:38, v/v) with a simulated intestinal fluid (SIF, 42.1 U trypsin/mL, pH 6.6, 8.2 mM bile, 10 mM KCl, 249 mM NaCl), followed by readjusting the pH to 6.6.

The adult *in vitro* gastrointestinal digestion was performed according to the method of Brodkorb et al. (2019) with slight modifications. This method was developed based on *in vivo* data derived from the gastrointestinal tract of healthy adult (Bohn et al., 2018). For gastric digestion, each casein micelle dispersion was mixed (50:50, v/v) with a SGF (4000 U pepsin/mL, pH 3.0, 6.9 mM KCl, 0.9 mM KH_2PO_4, 72.2 mM NaCl, 0.1 mM $MgCl_2$, 0.5 mM $(NH_4)_2CO_3$), followed by readjusting the pH to 3.0. After gastric digestion, the digesta were adjusted to pH 7.0 using dropwise addition of 2 M NaOH, and then mixed (50:50, v/v) with a SIF (200 U trypsin/mL, pH 7.0, 20 mM bile, 6.8 mM KCl, 0.8 mM KH_2PO_4, 123.4 mM NaCl, 0.33 mM $MgCl_2$), followed by readjusting the pH to 7.0.

The elderly *in vitro* gastrointestinal digestion was performed according to the methods of Levi and Lesmes (2014), and Mackie et al. (2020) with some modifications. This method was developed based on *in vivo* data derived from the gastrointestinal tract of healthy elderly aged ≥65 years (Hernández-Olivas et al., 2020). For gastric digestion, each casein micelle dispersion was mixed (50:50, v/v) with a SGF (3000 U pepsin/mL, pH 4.0, 6.9 mM KCl, 0.9 mM KH_2PO_4, 72.2 mM NaCl, 0.1 mM $MgCl_2$, 0.5 mM $(NH_4)_2CO_3$), followed by readjusting the pH to 4.0. After gastric digestion, the digesta were adjusted to pH 7.0 using dropwise addition of 2 M NaOH, and then mixed (50:50, v/v) with a SIF (92 U trypsin/mL, pH 7.0, 10.7 mM bile, 6.8 mM KCl, 0.8 mM KH_2PO_4, 123.4 mM NaCl, 0.33 mM $MgCl_2$), followed by readjusting the pH to 7.0.

Digesta samples were collected at 0.5, 2, 5, 10, 20, 30 and 60 min during both the gastric and intestinal digestion, and pepstatin A (7.3 μM) and Pefabloc (5.0 mM) were added immediately to terminate the pepsinolysis and trypsinolysis, respectively. Gastric digesta prepared as mentioned above but without added pepsin were the control sample collected at 0 min. Part of the gastric digesta was placed in a Petri-dish (35 mm in diameter) and photographed using an IXUS 210 digital camera (Canon Inc., Tokyo, Japan).

2.7. Confocal laser scanning microscopy (CLSM)

The gastric digesta were labeled using fluorescein isothiocyanate (2 μg/mL), and then placed into a glass bottom cell culture dish (Shengyou Biotechnology Co., Zhejiang, China), following the method of Liu et al. (2019). Visualization was done using a TCS SP8 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) at excitation and emission wavelengths of 488 and 498–532 nm, respectively. The micrographs were taken using a 10 × objective lens.

2.8. Determination of coagulum moisture content

The gastric digesta collected at 0.5 min of digestion were filtered using a 2-layered cloth, following the method of Li et al. (2022). The cake layer was dried at 105 °C for 7 h using a BGZ-30 oven (Boxun Industry & Commerce Co., Ltd., Shanghai, China), and the difference between the weights before and after drying, expressed as a percentage of the weight before drying, was defined as the coagulum moisture content.

2.9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done using a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) loaded with a 4% stacking gel and a 13% resolving gel according to the method of Liu et al. (2019). The digesta samples were diluted in ultrapure water to obtain an equivalent casein content of 2 g/L, and then mixed (1:1, v/v) with a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 25% (v/v) glycerol). The mixtures were boiled for 3 min, and then a 10 μL of sample was loaded in each well. After electrophoresis, the gels were stained using 0.1% (w/v) Coomassie Brilliant Blue R-250 for 4 h, and then destained using 7.5% (v/v) acetic acid and 5% (v/v) methanol until clear bands were visible. Semi-quantitative analysis of the band intensity was done using a Bio-Rad ChemiDoc XRS + Imager loaded with an Image Lab™ 3.0 software (Bio-Rad Laboratories, Inc.).

2.10. OPA spectrophotometric assay

Free amino groups in the digesta were determined according to the method of Torcello-Gómez et al. (2020) using L-leucine (0–3 mM) as the standard. The digesta samples were mixed (1:1, v/v) with 6.2% trichloroacetic acid, and then centrifuged at 10,000 ×*g* for 10 min. A 150 μL of the resulting supernatant was mixed with 3 mL of a reagent containing 0.08% (w/v) OPA, 3.8% (w/v) $Na_2B_4O_7$, 0.1% (w/v) SDS and 0.09% (w/v) dithiothreitol. After being incubated in the dark for 15 min, the absorbance was measured at 340 nm using the UV-2700 spectrophotometer.

2.11. High performance size exclusion chromatography

Molecular weight distribution of peptides in the digesta was measured using a Waters e2695 Separations Module (Waters Corp., Milford, MA, USA) loaded with a TSK-GEL G2000SWxl column (Tosoh Bioscience LLC, Montgomeryville, PA, USA), according to the method of Liu et al. (2019) with slight modification. The digesta samples were mixed (1:1, v/v) with 8 M urea, and then passed through syringe filters (nylon membrane, 0.45 μm pore size; Fuji Science & Technology Co., Ltd., Tianjin, China). A 50 μL aliquot of each filtrate was injected, and eluted using a solvent of acetonitrile-water-trifluoroacetic acid (400:600:1, v/v/v) at a flow rate of 0.5 mL/min. To estimate the molecular weight, the cytochrome C, bacitracin, Gly-Gly-Tyr-Arg and Gly-Gly-Gly were used as the standards.

2.12. Statistical analysis

Statistical analysis was done using an 8th version statistical analysis system (SAS Institute, Inc., Cary, NC, USA). One-way analysis of variance with the General Linear Model procedure was performed and the differences between means were determined with the Duncan's test at a significance level of 0.05. Principal component analysis (PCA) was performed using a XL STAT 2016 software (Addinsoft Inc., New York, USA).

3. Results and discussion

3.1. Ca distribution and turbidity of casein micelle dispersions

With increasing level of added ion-exchange resin, more Ca was removed; i.e., the amounts of total, permeable and non-permeable Ca decreased gradually in the bovine casein micelle dispersions (Table 1). The level of decalcification achieved for the micelle dispersions using 1.1, 3.8, 8.0 and 16.0 g resin/100 g retentate were ~18, 40, 69 and 90%, respectively. Consequently, the relative turbidity of the micelle dispersions decreased sharply to 0.16 with increasing level of decalcification up to 40% and then decreased slightly to 0.09 as the level of decalcification further increased to 90% ($P < 0.05$), suggesting an almost complete disintegration of the micellar structures at and above 40% decalcification. Xu et al. (2016) also reported that at ≥ 38.7% decalcification, complete disintegration of casein micelles in milk protein concentrate into smaller aggregates was observed using transmission electron microscopy. The amount of non-permeable Ca for non-decalcified bovine micelles was 31.9 mg/g casein (Table 1); while Yang, Liu, and Zhou (2022) reported that for human casein micelles, the amount of non-permeable Ca was 13.4 mg/g casein, corresponding equivalently to that of the bovine casein micelles with 58.0% decalcification where, however, the bovine micelles completely disintegrated, due probably to a higher phosphorylation level for the bovine caseins. The micelle dispersion with 90% decalcification had a total Ca content of 3.8 mg/g casein, which is close to the total Ca content of 3.4 mg/g casein for a commercial sodium caseinate according to the manufacturer's specification (Fonterra Co-operative Group Ltd., Auckland, New Zealand).

3.2. Gastric digestion

3.2.1. Coagulation behaviors

The morphology of the coagulum formed during the *in vitro* gastric digestion of casein micelles under the infant condition are shown in Fig. 1. At 0% decalcification, large and dense clusters were formed upon decreasing the pH to 5.3 at 0 min, and then fragmented gradually and slightly to form smaller clusters after adding pepsin. At 18% decalcification, small fragmented clusters were formed at 0 min and then grew gradually and slightly in size with increasing time. At 40% decalcification, macroscopically visible flocs were formed at 0 min and then grew gradually in size with time. At both 69 and 90% decalcification, no visible coagulum was observed at 0 min, and then looser macroscopic flocs were formed at 0.5 min and thereafter grew markedly in size with time. This differed from the findings by Huppertz and Lambers (2020) who reported that during an infant *in vitro* dynamic gastric digestion of a model infant formula, small clusters were formed for the bovine micelles without decalcification, while no visible clusters were formed for the bovine micelles with 13–63% decalcification. The less extensive gastric coagulation for bovine micelles in the model infant formula might be partially due to the presence of whey proteins, which could act as inert barriers between the micelles (Yang, Ye, et al., 2022).

For the *in vitro* gastric digestion under the adult condition (Fig. 2), at 0–40% decalcification, large and dense clusters with decreasing sizes were formed upon decreasing the pH to 3.0 at 0 min, and then remained almost unchanged with increasing time after adding pepsin. At 69–90% decalcification, no visible coagulum was observed at 0 min, and then microscopically visible flocs were formed at 0.5 min and thereafter grew slightly in size with time. This differed from the findings by Wang et al. (2018) who reported that during an adult *in vitro* dynamic gastric digestion, the clusters formed from the micelles in bovine milk protein concentrate (MPC) with 42% decalcification were looser compared to that without decalcification, and were generally similar to those formed from sodium caseinate at a same protein concentration. While in the present study, a denser coagulum was formed at 40% decalcification than that at 90% decalcification where the total Ca content was similar to that of a commercial sodium caseinate (Fonterra Co-operative Group Ltd.). The presence of whey proteins in MPC would inhibit the inter-micellar cross-linking, while the remaining calcium in MPC would promote the inter-micellar cross-linking, thus resulting in the similar gastric coagulation behaviors between MPC with 42% decalcification and caseinate (Yang, Ye, et al., 2022). Under the elderly condition (Fig. 3), at 0–40% decalcification, large and dense clusters were formed upon decreasing the pH to 4.0 at 0 min, and then fragmented gradually into small clusters after adding pepsin. At 69–90% decalcification, looser macroscopic flocs were formed at 0 min and then decreased gradually in size with increasing time.

The moisture contents of coagula formed at 0.5 min of *in vitro* gastric digestion of casein micelles are shown in Table S2. Under each age condition, the coagulum moisture generally increased with increasing level of decalcification, indicating an increasingly looser structure of the coagulum; while at each level of decalcification, the coagulum moisture generally increased in order of adult, elderly and infant conditions ($P < 0.05$), except at 69–90% decalcification where no coagulum was obtained under the adult condition. Taken together the results regarding morphology and moisture, it was suggested that the gastric coagula of bovine micelles became increasingly looser with increasing level of decalcification, especially when reaching 40–90% under the infant condition and 69–90% under the adult and elderly conditions.

Upon gastric digestion of casein micelles, the decrease in pH induced the solubilization of colloidal Ca and the collapse of κ-casein hairy layers, and subsequently the proteolysis by pepsin caused the removal of κ-casein hairy layers, thus reducing the electrostatic and steric repulsions between micelles (Huppertz & Chia, 2021). The removal of κ-casein hairy layers also exposed the Ca sensitive caseins buried within micelles, thus further promoting the occurrence of inter-micellar Ca bridging (Yahimi-Yazdi, Corredig, & Dalgleish, 2014). These multiple effects contributed together to the formation of large and dense coagula during the gastric digestion of bovine micelles. With increasing level of decalcification of micelles, the micellar structures gradually disintegrated, and less colloidal Ca was solubilized upon decreasing pH, thus reducing the occurrence of Ca bridging and hence contributing to the formation of smaller and looser coagula during gastric digestion (Liao et al., 2022).

For the *in vitro* gastric digestion under the infant condition, the high pH used was above the isoelectric point of caseins (O'Mahony & Fox, 2013), which, together with the low level of pepsin that might cause a slow removal of κ-casein hairy layers, led to the formation of looser gastric coagulum. It was noted that at high level of decalcification, where the micellar structures were completely disintegrated, decreasing pH alone (i.e., without adding pepsin) was not enough to cause the initial coagulation. During digestion, the large peptides released might re-associate with remaining intact caseins, leading to the increase in size of the coagulum (Radosavljević et al., 2020). Under the adult condition,

Table 1
Calcium distribution and relative turbidity of the dispersions of casein micelles with 0–89.6% decalcification.

Level of decalcification (%)	Total Ca (mg/L)	Permeable Ca (mg/L)	Non-permeable Ca (mg/g casein)	Relative turbidity
0	406.5 ± 15.0^{a}	23.4 ± 0.2^{a}	31.9 ± 1.2^{a}	1.00 ± 0.01^{a}
17.5	330.6 ± 0.2^{b}	14.7 ± 0.4^{b}	26.3 ± 0.1^{b}	0.56 ± 0.01^{b}
40.2	239.9 ± 8.1^{c}	10.7 ± 0.2^{c}	19.1 ± 0.7^{c}	0.16 ± 0.01^{c}
69.3	127.2 ± 0.6^{d}	9.5 ± 0.3^{c}	9.8 ± 0.1^{d}	0.11 ± 0.00^{d}
89.6	46.2 ± 0.2^{e}	6.3 ± 1.7^{d}	3.3 ± 0.1^{e}	0.09 ± 0.01^{e}

$^{a\text{-}e}$ Means in a column followed by different lower-case letters differ significantly ($P < 0.05$).

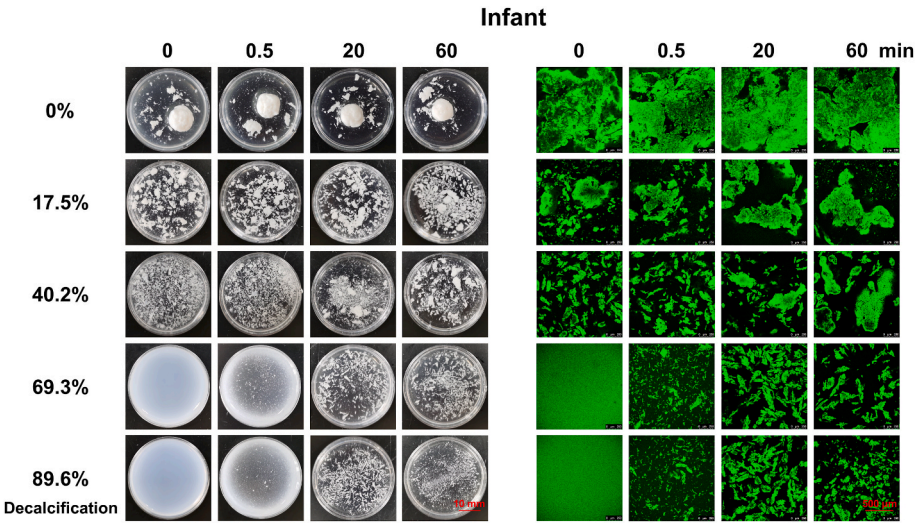

Fig. 1. Visual appearance (left) and CLSM micrographs (right) of the infant *in vitro* gastric digesta of casein micelles with 0–89.6% decalcification.

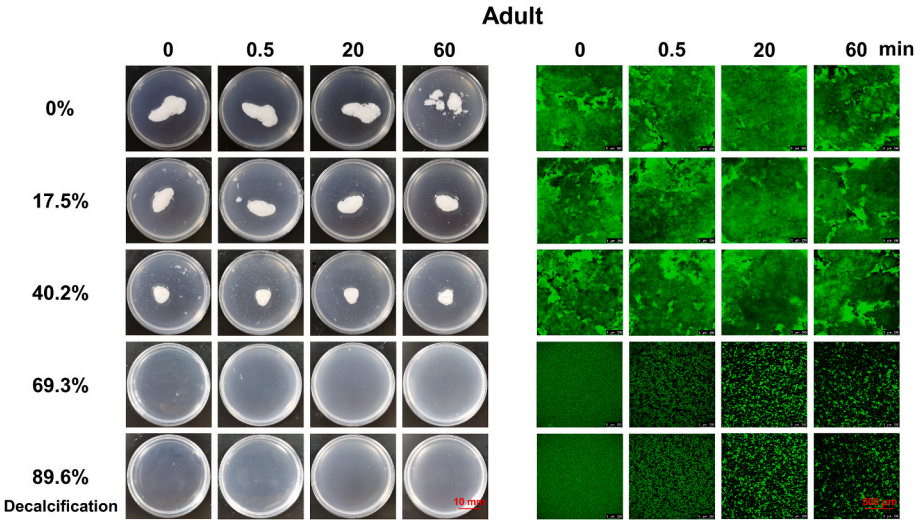

Fig. 2. Visual appearance (left) and CLSM micrographs (right) of the adult *in vitro* gastric digesta of casein micelles with 0–89.6% decalcification.

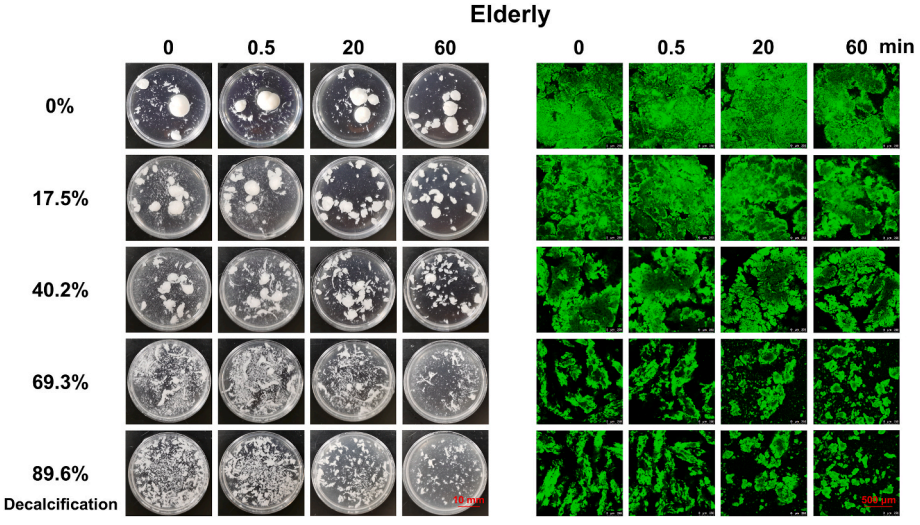

Fig. 3. Visual appearance (left) and CLSM micrographs (right) of the elderly *in vitro* gastric digesta of casein micelles with 0–89.6% decalcification.

the high level of pepsin might cause a rapid removal of κ-casein hairy layers, and hence led to the formation of denser gastric coagulum at low decalcification (Wang et al., 2018; Ye et al., 2019). The high level of pepsin, together with the high decalcification, might lead to rapid degradation of caseins and hence the initial absence of coagulum. Moreover, the low pH of 3 induced almost complete solubilization of non-permeable Ca, i.e., disruption of Ca bridges, thus further leading to the initial absence of coagulum (Broyard & Gaucheron, 2015). During digestion, the large peptides released might self-aggregate, contributing to the appearance of microscopic flocs (Radosavljević et al., 2020). Under the elderly condition, the pH used was close to (i.e., slightly below) the isoelectric point of caseins, which, together with the moderate level of pepsin, contributed to the formation of relatively denser gastric coagulum. During digestion, the degradation of caseins, together with the mechanical agitation, contributed to the decrease in size of the coagulum.

3.2.2. Proteolysis behaviors

The degradation of caseins during the *in vitro* gastric digestion of casein micelles is shown in Fig. 4. Under the infant condition, at 0 and 18% decalcification, the percentages of intact caseins decreased gradually and remained at 56–63% at the end of gastric digestion; while at 40–90% decalcification, the percentages of intact caseins decreased rapidly and remained at 24–29% at the end of gastric digestion ($P < 0.05$). Under the adult condition, at 0–40% decalcification, the percentages of intact caseins decreased rapidly and remained at 23–42% at the end of digestion ($P < 0.05$). This differed from the findings by Zhang et al. (2023) who reported that during an adult *in vitro* dynamic gastric digestion of bovine skim milk, the intact caseins disappeared completely after 60 min. The faster casein degradation might be partially due to the formation of a looser gastric cluster with the presence of whey proteins. It was noted that the degradation of intact κ-casein was even more rapid, i.e., the band intensity was negligible after 0.5–2 min of digestion, which agreed with the formation of dense gastric coagulum as explained above (Fig. 2 and Table 1). At 69 and 90% decalcification, the intact caseins disappeared completely after only 0.5–2 min of gastric digestion, which agreed with the initial absence of coagulum as explained above (Fig. 2). Under the elderly condition, at 0–40% decalcification, the percentages of intact caseins decreased gradually and remained at 45–69% at the end of gastric digestion; while at 69 and 90% decalcification, the percentages of intact caseins decreased rapidly and remained at 12–13% at the end ($P < 0.05$). Together with the degradation of intact caseins was the release of large peptides, with the 15–20 and < 10 kDa fractions appearing most abundant under the infant and adult conditions, respectively, which agreed with the increase in size of the coagula during digestion as explained above (Figs. 1 and 2).

The formation of free amino groups during the *in vitro* gastric digestion of casein micelles is shown in Fig. 5. Under the infant condition, the amounts of free amino groups increased slightly with time, indicating an increasing level of proteolysis; while under the adult condition, the amounts of free amino groups increased sharply within the initial 5 min, followed by a gradual increase until the end ($P < 0.05$). Under the elderly condition, the amounts of free amino groups increased steadily with time ($P < 0.05$). At the end of gastric digestion, under each age condition, the amounts of free amino groups generally increased with increasing level of decalcification; while at each decalcification, the amounts of free amino groups generally increased in order of infant, elderly and adult conditions ($P < 0.05$). These results generally agreed with previous researches on *in vitro* gastric digestion of bovine milk, where a faster proteolysis was obtained under an adult condition than under an infant (Torcello-Gómez et al., 2020) or elderly (Zhang et al., 2023) condition.

The molecular weight distribution of peptides formed at 60 min of *in vitro* gastric digestion of casein micelles is shown in Fig. 6. With increasing level of decalcification, under the infant condition, the abundance of the >10 kDa fraction decreased gradually with a concomitant increase in the abundance of all the 5–10, 1–5 and <1 kDa fractions, indicating an increasing level of proteolysis; while under the adult condition, the abundance of both the >10 and 5–10 kDa fractions decreased slightly with a concomitant increase in the abundance of both the 1–5 and <1 kDa fractions ($P < 0.05$). With increasing level of decalcification, under the elderly condition, the abundance of both the >10 and 5–10 kDa fractions decreased gradually with a concomitant increase in the abundance of both the 1–5 and <1 kDa fractions ($P < 0.05$). At each decalcification, the abundance of the <5 kDa fractions, i.e., small peptides below the detection limits of the present electrophoresis, generally increased in order of infant, elderly and adult conditions ($P < 0.05$). These results agreed with the formation of free amino groups at the end of digestion as a function of decalcification and age populations (Fig. 5). Taken together the results regarding casein degradation followed by formation of free amino groups and peptides, it was suggested that the gastric proteolysis of bovine micelles became increasingly faster with increasing level of decalcification, especially up to 40% under the infant condition and 69% under the adult and elderly conditions.

The gastric proteolysis of casein micelles was largely affected by their coagulation behaviors and the pepsin levels (Huppertz & Lambers, 2020; Wang et al., 2018). Under the infant, adult and elderly conditions, the structures of gastric coagula became characterized by markedly smaller particles and looser particle structures at ≥ 40%, ≥69% and ≥69% decalcification (Figs. 1–3, Table 1), respectively, where a marked improvement in both the rate of intact casein degradation and the level of proteolysis was achieved correspondingly (Figs. 4–6), suggesting that the smaller and looser coagula with increasing level of decalcification increased the accessibility of pepsin to casein peptide bonds (Huppertz & Lambers, 2020). These results agreed with Nelson, Lynch, and Barbano (2004) who reported that compared to a cheddar cheese produced from untreated milk, the one produced from milk pre-acidified with CO_2 showed a higher content of Ca and a faster casein proteolysis upon aging, due probably to an increased substrate availability in the cheese matrix. The formation of small and loose gastric casein coagula was suggested to expedite the gastric emptying and the amino acid/peptide absorption, especially for the infants and elderly who had a low activity of digestive proteases in their gastrointestinal tract (Chan et al., 2019; Roy et al., 2022; Ye et al., 2019).

For the *in vitro* gastric digestion under adult condition, the low pH used was close to the optimal pH for pepsin activity, which, together with the high level of pepsin added, led to both the highest rate of intact casein degradation and the highest level of gastric proteolysis among the three age conditions (Dupont et al., 2010; Salelles, Floury, & Le Feunteun, 2021). At 0–40% decalcification, under the elderly condition, the gastric coagulum of micelles was markedly denser compared to the infant condition, leading to the lower rate of casein degradation. However, it was noted that under the infant condition, the level of gastric proteolysis was lower (Figs. 5 and 6) and the abundance of large (15–20 kDa) peptides released was higher (Fig. 4), probably because the pH used was farther above the optimal pH for pepsin activity and the level of pepsin added was much lower compared to the elderly condition. At 69–90% decalcification, under the infant condition, the high pH used and the low level of pepsin added led to both the lower rate of casein degradation and the lower level of gastric proteolysis. The gastric pH used under the infant, elderly and adult conditions was 5.3, 4.0 and 3.0, where the ratio of pepsin activity was 7:11:37, respectively. Taken together with the level of pepsin added, the actual pepsin activity used under the infant, elderly and adult conditions was ~19, 165 and 740 U/mL, respectively, leading to the correspondingly increasing level of gastric proteolysis (Figs. 5 and 6). With a same actual pepsin activity at the different pH used under the infant, elderly and adult conditions, the gastric coagulum of micelles became increasingly denser while the formation of free amino groups decreased correspondingly, suggesting that a looser coagulum would increase the accessibility of pepsin to casein peptide bonds (Fig. S1).

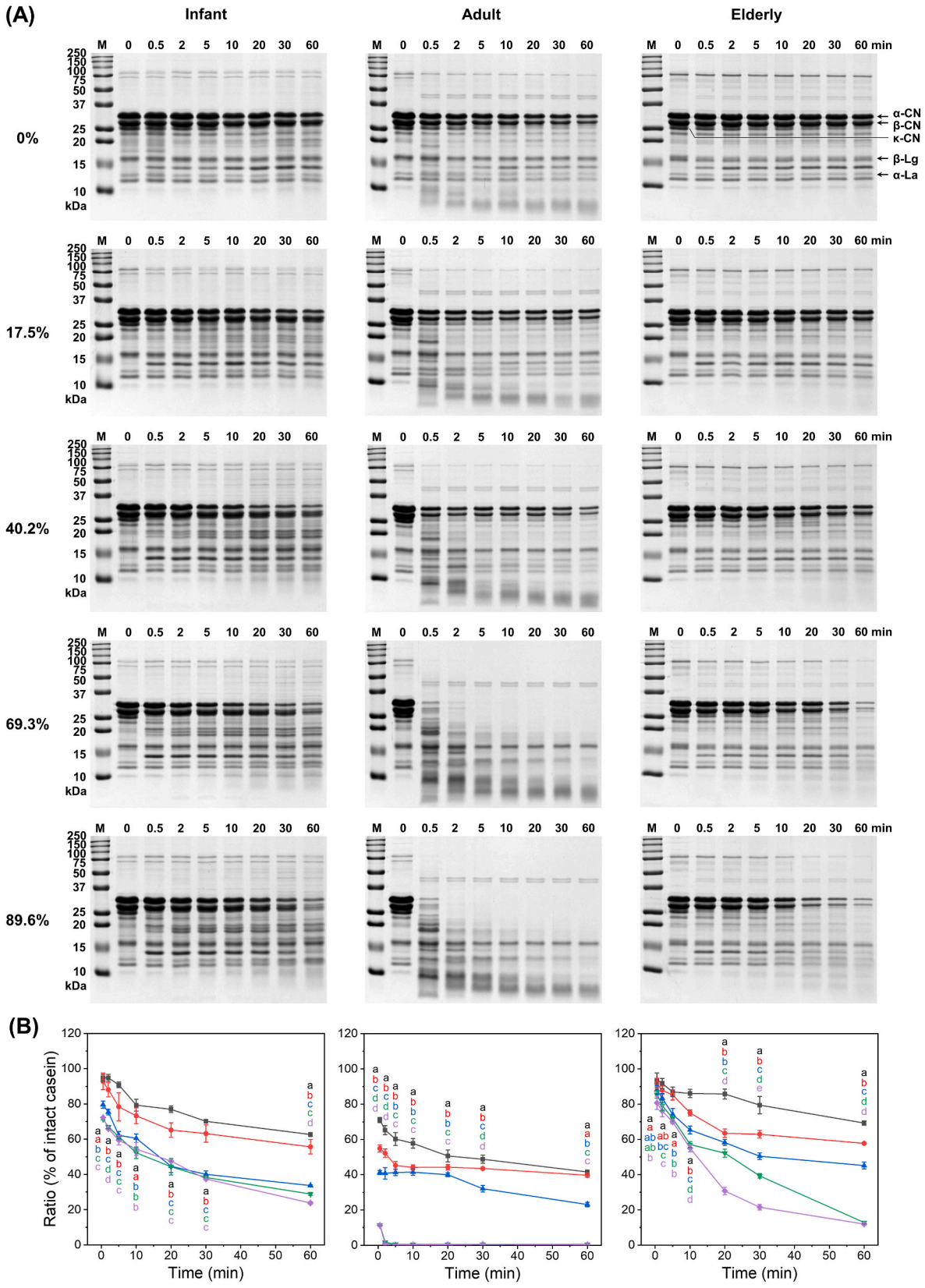

Fig. 4. SDS-PAGE patterns (A) and percentages of remaining intact caseins (B) of the infant, adult and elderly *in vitro* gastric digesta of casein micelles with 0–89.6% decalcification (■, 0%; ●, 17.5%; ▲, 40.2%; ▼, 69.3%; ◆, 89.6%). Different lower-case letters indicate that the data differ significantly among different level of decalcification at a same digestion time ($P < 0.05$); M, protein markers; CN, casein; Lg, β-lactoglobulin; La, α-lactalbumin.

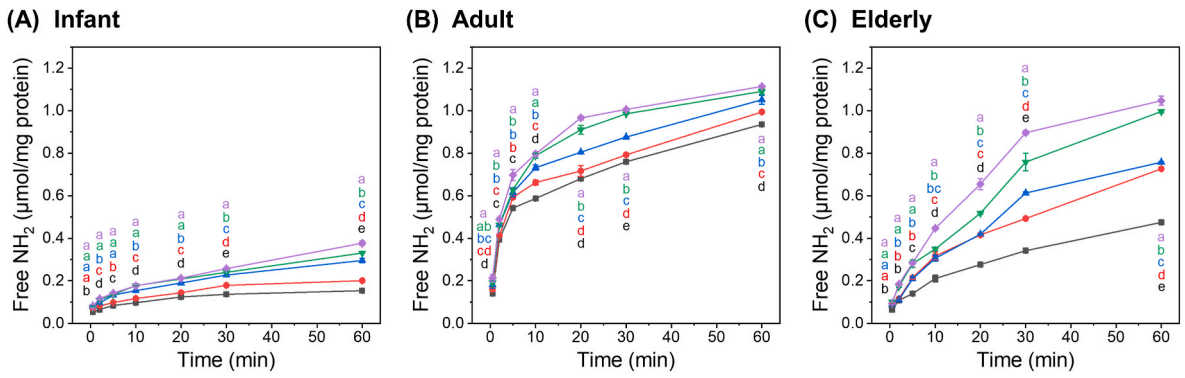

Fig. 5. Concentration of free amino groups in the infant (A), adult (B) and elderly (C) *in vitro* gastric digesta of casein micelles with 0–89.6% decalcification (■, 0%; ●, 17.5%; ▲, 40.2%; ▼, 69.3%; ◆, 89.6%). Different lower-case letters indicate that the data differ significantly among different level of decalcification at a same digestion time ($P < 0.05$).

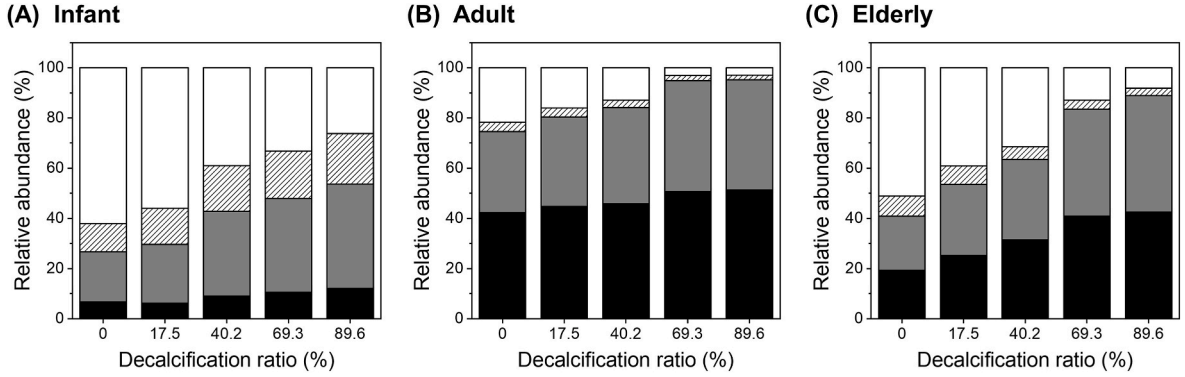

Fig. 6. Molecular weight distribution (□, >10 kDa; ▨, 5–10 kDa; ▩, 1–5 kDa; ■, <1 kDa) of peptides in the infant (A), adult (B) and elderly (C) *in vitro* gastric digesta (60 min) of casein micelles with 0–89.6% decalcification.

To better understand the factors affecting the gastric proteolysis of bovine micelles under three *in vivo*-relevant conditions, a PCA score biplot (Fig. S2) was established based on the percentage of remaining intact caseins (Fig. 4B), the concentration of free amino groups (Fig. 5) and the molecular weight distribution of peptides (Fig. 6). The first and second principal components account for 79.7% and 11.9% of the total variance, respectively, thus well explaining the variability in gastric proteolysis of micelles. The three age conditions were well separated along the first principal component, and the different level of decalcification was well separated along the second principal component. Well separation was also observed up to 40% decalcification for the infant condition and up to 69% decalcification for the adult and elderly conditions. The three age conditions differed in pH and pepsin levels, which affected both the micelle coagulation and the pepsin activity, and the amount of Ca in micelles affected their coagulation. Taken together, these results suggested a combined effect of coagulation behavior and pepsin activity on the gastric proteolysis of bovine micelles, and that decalcification up to 40% for the infant and up to 69% for the adult and elderly were suggested to markedly improve the proteolysis of bovine micelles.

3.3. Intestinal digestion

At the end of gastric digestion of casein micelles, under the three age conditions, no marked differences were observed in the coagulum structures or the level of proteolysis between 69 and 90% decalcification. Therefore, after gastric digestion, the digesta of casein micelles with 0, 18, 40 and 69% decalcification were used for subsequent intestinal digestion, where the remaining intact caseins were very rapidly degraded under the three age conditions, i.e., the band intensity was negligible after only 0.5 min of digestion (Fig. S3). Torcello-Gómez et al. (2020) also reported that the remaining intact caseins after infant *in vitro* gastric digestion of fresh whole milk were degraded completely after only 0.5 min of subsequent intestinal digestion.

During *in vitro* intestinal digestion under the three age conditions, the amounts of free amino groups continued to increase gradually ($P < 0.05$) (Fig. 7). Under the infant condition, the amounts of free amino groups were slightly larger for the decalcified micelles than for the non-decalcified micelles within the initial 30 min of digestion ($P < 0.05$), while no significant differences were observed among different level of decalcification at the end of digestion ($P > 0.05$). Under both adult and elderly conditions, throughout the digestion, no significant differences in the amounts of free amino groups were observed among different level of decalcification ($P > 0.05$). Throughout the digestion, the amounts of free amino groups generally increased in order of infant, elderly and adult conditions, indicating an increasing level of proteolysis ($P < 0.05$). Previous researches on *in vitro* intestinal digestion of bovine milk following a gastric digestion also reported that the proteolysis was faster under an adult condition than under an infant (Torcello-Gómez et al., 2020) or elderly (Hernández-Olivas et al., 2020) condition.

The molecular weight distribution of peptides released in the *in vitro* intestinal digesta of casein micelles is shown in Fig. 8. Under the three age conditions, the abundance of the >0.5 kDa fraction continued to decrease gradually with a concomitant increase in the abundance of the <0.5 kDa fraction during digestion ($P < 0.05$). Under the infant condition, the abundance of the <0.5 kDa fraction increased slightly with increasing level of decalcification within the initial 10 min of digestion ($P < 0.05$); while no marked differences were observed among different level of decalcification at the end of digestion. Under both the adult and elderly conditions, throughout the digestion, no marked differences in

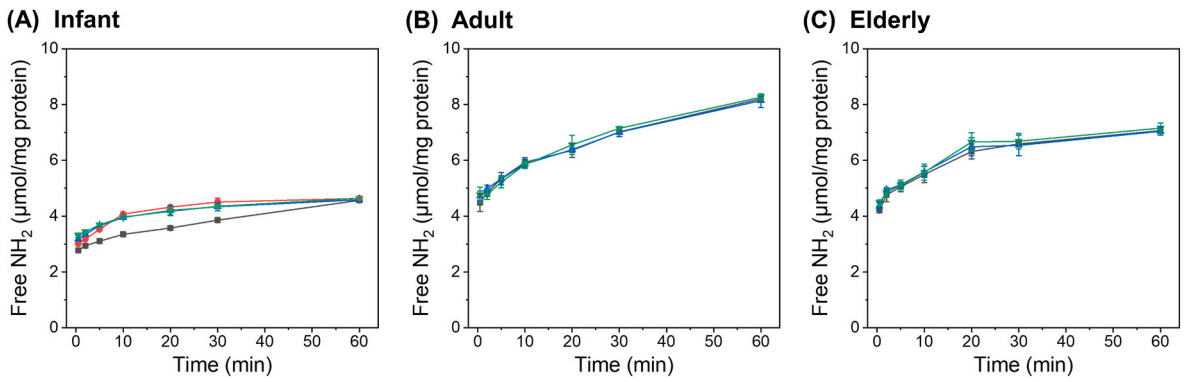

Fig. 7. Concentration of free amino groups in the infant (A), adult (B) and elderly (C) *in vitro* intestinal digesta of casein micelles with 0–69.3% decalcification (■, 0%; ●, 17.5%; ▲, 40.2%; ▼, 69.3%).

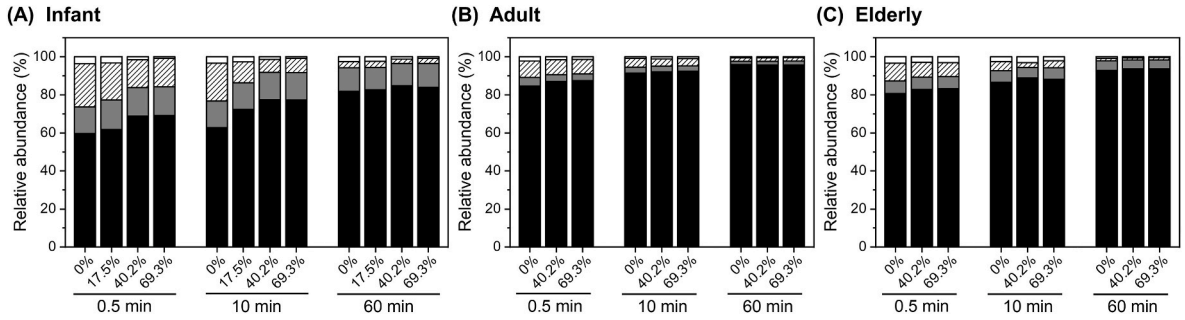

Fig. 8. Molecular weight distribution (□, >5 kDa; ▨, 1–5 kDa; ▩, 0.5–1 kDa; ■, <0.5 kDa) of peptides in the infant (A), adult (B) and elderly (C) *in vitro* intestinal digesta (0.5, 10 and 60 min) of casein micelles with 0–69.3% decalcification.

the abundance of the <0.5 kDa fraction were observed among different level of decalcification. Throughout the digestion, the abundance of the <0.5 kDa fraction generally increased in order of infant, elderly and adult conditions, indicating an increasing level of proteolysis ($P < 0.05$). These results generally agreed with the formation of free amino groups during digestion as a function of decalcification and age populations (Fig. 7).

Upon intestinal digestion, the increased pH induced the increase in inter-molecular electrostatic repulsions and the concomitant solubilization of the digesta, leading to an increased accessibility of trypsin to peptide bonds and hence the rapid proteolysis (Bouzerzour et al., 2012; Halabi et al., 2022). Under the three age conditions, the levels of digestive proteases added in the form of pancreatin increased in order of infant, elderly and adult, leading to an increasing level of proteolysis correspondingly. Under the infant condition, due to the low level of pancreatin added, differences in the level of proteolysis among different level of decalcification obtained after gastric digestion continued to exist within the initial 30 min of intestinal digestion. Under both the adult and elderly conditions, the high level of pancreatin added resulted in no marked differences in the level of proteolysis among different level of decalcification throughout intestinal digestion.

4. Conclusions

Decalcification of bovine casein micelles greatly affected their *in vitro* coagulation behaviors and gastrointestinal digestibility under infant, adult and elderly conditions. Gastric coagula became looser with increasing level of decalcification, especially up to 40% under the infant condition and up to 69% under the adult and elderly conditions, thus increasing casein degradation by reducing the limitation in the accessibility of digestive proteases to peptide bonds. Casein degradation was fastest under adult condition, and slowest under elderly and infant conditions at 0–40 and 69–90% decalcification, respectively. Formation of free amino groups and small peptides increased with increasing level of decalcification and in order of infant, elderly and adult conditions. During intestinal digestion, formation of free amino groups and smaller peptides showed marginal differences with increasing level of decalcification, and increased in order of infant, elderly and adult conditions. These results suggested a combined effect of coagulation behavior and protease activity in the gastrointestinal tract on the digestibility of bovine casein micelles, and that decalcification was an efficient approach to weaken the coagulation of bovine micelles and hence promote their gastrointestinal digestibility. In turn, bovine micelles with ~40% decalcification could be potentially applied, e.g., in the manufacture of infant formula, and those with ~69% decalcification could be used in sports and elderly nutrition supplements, to achieve an improved digestibility especially for those with maldigestion.

CRediT authorship contribution statement

Keyu Wang: Investigation, Methodology, Writing - original draft. **Dasong Liu:** Conceptualization, Writing - review & editing. **Xiumei Tao:** Methodology, Writing - review & editing. **Jie Zhang:** Methodology, Writing - review & editing. **Thom Huppertz:** Methodology, Writing - review & editing. **Joe M. Regenstein:** Methodology, Writing - review & editing. **Xiaoming Liu:** Methodology, Writing - review & editing. **Peng Zhou:** Conceptualization, Supervision, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2023.108515.

References

Aalaei, K., Khakimov, B., De Gobba, C., & Ahrné, L. (2021). Digestion patterns of proteins in pasteurized and ultra-high temperature milk using *in vitro* gastric models of adult and elderly. *Journal of Food Engineering, 292*, Article 110305.

Bohn, T., Carriere, F., Day, L., Deglaire, A., Egger, L., Freitas, D., et al. (2018). Correlation between *in vitro* and *in vivo* data on food digestion. What can we predict with static *in vitro* digestion models? *Critical Reviews in Food Science and Nutrition, 58*, 2239–2261.

Bourlieu, C., Ménard, O., Bouzerzour, K., Mandalari, G., Macierzanka, A., Mackie, A. R., et al. (2014). Specificity of infant digestive conditions: Some clues for developing relevant *in vitro* models. *Critical Reviews in Food Science and Nutrition, 54*, 1427–1457.

Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Huërou-Luron, I. L., et al. (2012). *In vivo* digestion of infant formula in piglets: Protein digestion kinetics and release of bioactive peptides. *British Journal of Nutrition, 108*, 2105–2114.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., et al. (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols, 14*, 991–1014.

Broyard, C., & Gaucheron, F. (2015). Modifications of structures and functions of caseins: A scientific and technological challenge. *Dairy Science & Technology, 95*, 831–862.

Chan, A. H., D'Souza, R. F., Beals, J. W., Zeng, N., Prodhan, U., Fanning, A. C., et al. (2019). The degree of aminoacidemia after dairy protein ingestion does not modulate the postexercise anabolic response in young men: A randomized controlled trial. *Journal of Nutrition, 149*, 1511–1522.

Dalgleish, D. G., & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Review of Food Science and Technology, 3*, 449–467.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Leonil, J., Faulks, R. M., et al. (2010). Comparative resistance of food proteins to adult and infant *in vitro* digestion models. *Molecular Nutrition & Food Research, 54*, 767–780.

Halabi, A., Croguennec, T., Ménard, O., Briard-Bion, V., Jardin, J., Gouar, Y. L., et al. (2022). Protein structure in model infant milk formulas impacts their kinetics of hydrolysis under *in vitro* dynamic digestion. *Food Hydrocolloids, 126*, Article 107368.

Hernández-Olivas, E., Muñoz-Pina, S., Sánchez-García, J., Andrés, A., & Heredia, A. (2020). Understanding the role of food matrix on the digestibility of dairy products under elderly gastrointestinal conditions. *Food Research International, 137*, Article 109454.

Huppertz, T., & Chia, L. W. (2021). Milk protein coagulation under gastric conditions: A review. *International Dairy Journal, 113*, Article 104882.

Huppertz, T., & Lambers, T. T. (2020). Influence of micellar calcium phosphate on *in vitro* gastric coagulation and digestion of milk proteins in infant formula model systems. *International Dairy Journal, 107*, Article 104717.

ISO. (2001). *Milk - determination of nitrogen content - Part 5: Determination of protein-nitrogen content*. Geneva, Switzerland: International Standardisation Organisation. *ISO 8968-5*.

ISO. (2004). *Milk - determination of casein-nitrogen content - Part 1: Indirect method*. Geneva, Switzerland: International Standardisation Organisation. *ISO 17997-1*.

Levi, C. S., & Lesmes, U. (2014). Bi-compartmental elderly or adult dynamic digestion models applied to interrogate protein digestibility. *Food & Function, 5*, 2402–2409.

Liao, M. J., Chen, F., Hu, X. S., Miao, S., Ma, L. J., & Ji, J. F. (2022). The *in-vitro* digestion behaviors of micellar casein acting as wall materials in spray-dried microparticles: The relationships between colloidal calcium phosphate and the release of loaded blueberry anthocyanins. *Food Chemistry, 375*, Article 131864.

Li, S. Q., Pan, Z., Ye, A. Q., Cui, J., Dave, A., & Singh, H. (2022). Structural and rheological properties of the clots formed by ruminant milks during dynamic *in vitro* gastric digestion: Effects of processing and species. *Food Hydrocolloids, 126*, Article 107465.

Liu, D. S., Zhang, J., Wang, L. L., Yang, T. Y., Liu, X. M., Hemar, Y., et al. (2019). Membrane-based fractionation, enzymatic dephosphorylation, and gastrointestinal digestibility of β-casein enriched serum protein ingredients. *Food Hydrocolloids, 88*, 1–12.

Mackie, A., Mulet-Cabero, A. I., & Torcello-Gómez, A. (2020). Simulating human digestion: Developing our knowledge to create healthier and more sustainable foods. *Food & Function, 11*, 9397–9431.

Ménard, O., Bourlieu, C., De Oliveira, S. C., Dellarosa, N., Laghi, L., Carriere, F., et al. (2018). A first step towards a consensus static *in vitro* model for simulating full-term infant digestion. *Food Chemistry, 240*, 338–345.

Nelson, B. K., Lynch, J. M., & Barbano, D. M. (2004). Impact of milk preacidification with CO_2 on the aging and proteolysis of cheddar cheese. *Journal of Dairy Science, 87*, 3590–3600.

Nurko, S., Benninga, M. A., Solari, T., & Chumpitazi, B. P. (2022). Pediatric aspects of nutrition interventions for disorders of gut-brain interaction. *American Journal of Gastroenterology, 117*, 995–1009.

O'Mahony, J. A., & Fox, P. F. (2013). Milk proteins: Introduction and historical aspects. In P. L. H. McSweeney, & P. F. Fox (Eds.), *Advanced dairy chemistry: Volume 1A: Proteins: Basic aspects* (4th ed., pp. 43–85). New York, New York: Springer Science+ Business Media.

Radosavljević, J., Apostolović, D., Mihailović, J., Atanasković-Marković, M., Burazer, L., van Hage, M., et al. (2020). Digestomics of cow's milk: Short digestion-resistant peptides of casein form functional complexes by aggregation. *Foods, 9*, 1576.

Roy, D., Moughan, P. J., Ye, A. Q., Hodgkinson, S. M., Stroebinger, N., Li, S. Q., et al. (2022). Structural changes during gastric digestion in piglets of milk from different species. *Journal of Dairy Science, 105*, 3810–3831.

Salelles, L., Floury, J., & Le Feunteun, S. (2021). Pepsin activity as a function of pH and digestion time on caseins and egg white proteins under static *in vitro* conditions. *Food & Function, 12*, 12468–12478.

Torcello-Gómez, A., Dupont, D., Jardin, J., Briard-Bion, V., Deglaire, A., Risse, K., et al. (2020). The pattern of peptides released from dairy and egg proteins is highly dependent on the simulated digestion scenario. *Food & Function, 11*, 5240–5256.

Wang, X., Ye, A. Q., Lin, Q. Q., Han, J. Z., & Singh, H. (2018). Gastric digestion of milk protein ingredients: Study using an *in vitro* dynamic model. *Journal of Dairy Science, 101*, 6842–6852.

Xu, Y. T., Liu, D. S., Yang, H. X., Zhang, J., Liu, X. M., Regenstein, J. M., et al. (2016). Effect of calcium sequestration by ion-exchange treatment on the dissociation of casein micelles in model milk protein concentrates. *Food Hydrocolloids, 60*, 59–66.

Yahimi-Yazdi, S., Corredig, M., & Dalgleish, D. G. (2014). Studying the structure of β-casein-depleted bovine casein micelles using electron microscopy and fluorescent polyphenols. *Food Hydrocolloids, 42*, 171–177.

Yang, T. T., Liu, D. S., & Zhou, P. (2022a). Temperature-dependent dissociation of human micellar β-casein: Implications of its phosphorylation degrees and casein micelle structures. *Food Chemistry, 376*, Article 131935.

Yang, M. X., Ye, A. Q., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2022b). Pepsin-induced coagulation of casein micelles: Effect of whey proteins and heat treatment. *Food Chemistry, 402*, Article 134214.

Ye, A. Q., Liu, W. L., Cui, J., Kong, X. N., Roy, D., Kong, Y. Y., et al. (2019). Coagulation behaviour of milk under gastric digestion: Effect of pasteurization and ultra-high temperature treatment. *Food Chemistry, 286*, 216–225.

Zhang, P., Iqbal, S., Deng, R. P., Duan, X., Han, H. X., Chen, X. D., et al. (2023). Impact of elderly gastrointestinal alterations on gastric emptying and enzymatic hydrolysis of skim milk: An *in vitro* study using a dynamic stomach system. *Food Chemistry, 402*, Article 134365.

Zou, Z. Z., Duley, J. A., Cowley, D. M., Reed, S., Arachchige, B. J., Koorts, P., et al. (2022). Digestibility of proteins in camel milk in comparison to bovine and human milk using an *in vitro* infant gastrointestinal digestion system. *Food Chemistry, 374*, Article 131704.