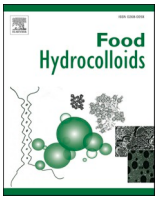
Food Hydrocolloids 139 (2023) 108550

Contents lists available at ScienceDirect

Food Hydrocolloids

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



Effect of ingestion temperature on the pepsin-induced coagulation and the *in vitro* gastric digestion behavior of milk

Mengxiao Yang [a], Aiqian Ye [a,*], Zhi Yang [b], David W. Everett [a,c], Elliot Paul Gilbert [d,e], Harjinder Singh [a]

[a] Riddet Institute, Massey University, Private Bag 11 222, Palmerston North, 4442, New Zealand
[b] School of Food and Advanced Technology, Massey University, Auckland, 0632, New Zealand
[c] AgResearch, Tennent Drive, Private Bag 11 008, Palmerston North, 4442, New Zealand
[d] Australian Centre for Neutron Scattering, ANSTO, New Illawarra Road, Lucas Heights, NSW, 2234, Australia
[e] Australian Institute for Bioengineering and Nanotechnology and Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, QLD, Australia

ARTICLE INFO

Keywords:
Temperature
Pepsin hydrolysis
Milk coagulation
Milk digestion

ABSTRACT

Pepsin-induced protein coagulation occurs in the gastric environment when the milk pH is above the isoelectric point of casein proteins. In this study, the effect of milk temperature (4–48 °C) on the hydrolysis of κ-casein by pepsin and the consequent protein coagulation was studied at pH 6.0 for 120 min. Quantitative determination of the released para-κ-casein showed that both the κ-casein hydrolysis reaction rate constant and the pepsin denaturation rate constant increased with an increase in temperature. The temperature coefficient (Q_{10}) of the specific hydrolysis of κ-casein was calculated to be ~1.95. The coagulation process was investigated by the evolution of the storage modulus (G'). At higher temperature, the milk coagulated faster but had a lower firming rate and G'_{max} with larger aggregates and voids were observed. The digestion behavior of the milk ingested at 4 °C, 37 °C, or 50 °C was investigated for 240 min in a human gastric simulator, in which the milk temperature increased or decreased to 37 °C (body temperature) over ~ 60 min. The coagulation of the 4 °C milk was slower than for the 37 °C and 50 °C milk. The curd obtained from the 4 °C milk had a looser and softer structure with a significantly higher moisture content at the initial stage of digestion (20 min) which, in turn, facilitated the breakdown and hydrolysis of the caseins by pepsin. During the digestion, the curd structure became more cohesive, along with a decrease in moisture content. The knowledge gained from this study provides insight into the effect of temperature on the kinetics of pepsin-induced milk coagulation and the consequent digestion behavior.

1. Introduction

Milk protein coagulation, including the specific hydrolysis of κ-casein and protein aggregation, is the initial step during gastric digestion of milk. Pepsin-induced hydrolysis and the coagulation behavior of the casein micelles in cows' skim milk at 37 °C have been extensively investigated (Ye, Cui, Dalgleish, & Singh, 2016, 2017; Huppertz & Chia, 2021; Yang et al., 2022; Ye et al., 2019). Depending on their drinking habits, some people like to drink warm milk, whereas some prefer to drink cold milk. As temperature is known to influence pepsin activity (Zhao, Budge, Ghaly, Brooks, & Dave, 2011) and enzyme-induced coagulation behavior (Dalgleish, 1983; Horne & Lucey, 2014; Nájera, De Renobales, & Barron, 2003), the temperature of the milk could influence the structure of the curd/clot that is induced by pepsin. The emptying of the stomach contents and release of nutrients would be expected to depend on the structural properties of the milk curd.

Pepsin hydrolyzes κ-casein specifically at the Phe^{105}–Met^{106} bond at pH > 5 with an optimum at pH ~6.0, which is similar to the action of chymosin on κ-casein (Yang et al., 2022). The reaction rate constant for the chymosin-induced hydrolysis of κ-casein increases with an increase in temperature from 7 to 37 °C (Turhan & Mutlu, 1998). The temperature coefficient (Q10), defined as the rate ratio of a given process taking place at temperatures differing by 10 units (°C or K), was used as a

* Corresponding author.
E-mail address: a.m.ye@massey.ac.nz (A. Ye).

https://doi.org/10.1016/j.foodhyd.2023.108550
Received 22 November 2022; Received in revised form 30 January 2023; Accepted 1 February 2023
Available online 1 February 2023

measure of the temperature sensitivity of chymosin-induced hydrolysis of κ-casein by Van Hooydonk (1987). These authors reported that Q_{10} varied between 1.3 and 2.0 and that hydrolysis occurred even at 0 °C. However, the effect of temperature on pepsin-induced hydrolysis of κ-casein has not been fully explored.

The aggregation phase of para-casein micelles occurs due to hydrophobic association and a decrease in electrostatic repulsion after a certain level of (glyco)macropeptide from κ-casein has been removed. The effect of temperature on the chymosin-induced aggregation phase has been investigated (Dalgleish, 1983; Gunasekaran & Ay, 1996; Horne & Lucey, 2014; Nájera et al., 2003). A greater effect of temperature has been found on the aggregation phase, with a Q_{10} of ~16 (Fox, Guinee, Cogan, & McSweeney, 2017, pp. 185–229). Aggregation occurs very slowly, or not at all, at temperatures <15 °C (Fox et al., 2017, pp. 185–229); the velocity of curd formation increases progressively from 20 to 42 °C but slows down at much higher temperatures because of denaturation of chymosin (Nájera et al., 2003). Mellema, Walstra, Van Opheusden, and Van Vliet (2002) demonstrated that rearrangement of the curd network structure was facilitated at higher temperature, resulting in soft curds at 35–36 °C, whereas hard or semihard curds formed at 31–32 °C. Panthi et al. (2019) and Ong, Dagastine, Kentish, and Gras (2011) reported that set temperature had a significant effect on the formation of curd microstructure; the casein micelles appeared to be smaller and less crosslinked in curds coagulated at a lower temperature (28 °C) than at a higher temperature (36 °C). In addition, after coagulation, syneresis was reported to occur more extensively at the higher temperature (Panthi et al., 2019).

According to Sun, Houghton, Read, Grundy, and Johnson (1988), temperature, *i.e.*, 4 °C, 37 °C, or 50 °C, impacts the rate of gastric emptying of a radiolabeled isosmotic drink of orange juice in humans. The intragastric temperature returned to body temperature within 20–30 min of ingestion of the 4 °C and 50 °C drinks, which appeared to empty from the stomach more slowly than the 37 °C drink. However, Webber, Nouri, and Bell (1980) found that cold meals increased the rate of gastric emptying in a study on calves, which was associated with increased acid and pepsin secretion. Despite the importance of temperature on the coagulation of milk proteins, no reports on the effect of temperature of milk on its digestion behavior have been published; therefore, an understanding of the interactive effects of milk temperature on the hydrolysis of κ-casein, the consequent coagulation, the curd structure, and the subsequent digestion behavior is desirable.

The objective of this study was to investigate the pepsin-induced protein hydrolysis and aggregation characteristics of skim milk (pH ~ 6.0, which is the optimum pH for the hydrolysis of κ-casein) at different temperatures. In addition, the different digestion behavior of the milk ingested at 4 °C, 37 °C, or 50 °C was investigated using a dynamic *in vitro* gastric model, the human gastric simulator (HGS) (Kong & Singh, 2010). The knowledge gained provides better understanding of the effect of drinking temperature on the coagulation and digestion of milk in a gastric environment.

2. Materials and methods

2.1. Materials

Fresh bovine milk was obtained from Dairy Farm 4 (Massey University, Palmerston North, New Zealand) and was skimmed by centrifugation with a swing bucket rotor (Thermo Fisher Scientific Multifuge Heraeus 3SR + centrifuge, Thermo Electron LED GmbH, Langenselbold, Germany) at 3000 *g* and 4 °C for 15 min. The skimmed milk contained 0.1% fat, 4.3% protein and 4.7% lactose as determined by a MilkoScan FT120 (Foss Electric, Hillerød, Denmark). The pH of the skim milk samples was measured to be ~6.7 at room temperature. Sodium azide was added at a concentration of 0.02% (wt/vol) to act as a bacteriostatic agent and stored for up to five days at 4 °C before experiments.

Pepsin (EC 3.4.23.1) from porcine gastric mucosa with an enzymatic activity of 550 U (mg protein)$^{-1}$ was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals (analytical grade) were also obtained from Sigma-Aldrich unless otherwise specified. Simulated salivary fluid (SSF, pH 7.0) and simulated gastric fluid (a 1.25 × concentrated electrolyte SGF, pH 1.5) were prepared according to Brodkorb et al. (2019).

2.2. Measurement of pepsin-induced milk hydrolysis and coagulation

The pH of skim milk samples was adjusted to 6.0 at 25 °C, by the gradual addition of SGF (1 × concentrate, pH 1.5) under vigorous stirring conditions (Yang et al., 2023). Different temperature profiles were applied by using a cold room, a water bath, an oven or a rheometer cup: (1) the temperatures were maintained at 4, 15, 25, 37, 40, 43, and 48 °C for 120 min; (2) increased from 4 to 37 °C or decreased from 50 to 37 °C in 30 min and kept at 37 °C for 90 min (these last two samples were designated as C4 °C and W50 °C milk, respectively, to distinguish them from the samples held at a constant temperature of 4 °C). The pH of samples at each temperature did not change more than 0.1 unit. Porcine pepsin (2 mg) was dissolved in Milli-Q water (10 mL; 110 U mL^{-1}). The diluted pepsin solutions were added to the skim milk samples at a ratio of 10 μL per 1 mL of milk and resulted in a final pepsin concentration of 1.10 U (mL milk)$^{-1}$. Hydrolysis and aggregation occurred within 120 min at this pepsin concentration.

2.2.1. Measurement of κ-casein hydrolysis

RP-HPLC was used to quantify the release of para-κ-casein (Yang et al., 2022). A 20 μL aliquot of diluted pepsin solution was added to the skim milk sample (2 mL), which was then immediately transferred into nine different test tubes, with 0.2 mL per tube, in a cold room or a water bath at each desired temperature. HPLC buffer solution (0.8 mL: 6 *M* guanidinium hydrochloride, 0.1 *M* bis-Tris, 19.5 m*M* dl-dithiothreitol, and 5.37 m*M* sodium citrate, pH 7) was subsequently added into each tube at different time points (1, 2, 5, 10, 20, 30, 60, 90, and 120 min) to stop the pepsin reaction. Each sample was shaken for 30 s, incubated for 1 h at room temperature and centrifuged before HPLC injection.

RP-HPLC analysis was carried out using a Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). Separation was carried out using a Phenomenex Aeris WIDEPORE XB-C18 column (100 mm × 4.6 mm, 3.6 μm particles). The column temperature was maintained at 45 °C and the detection wavelength was 214 nm. Chromatographic runs were carried out with an injection volume of 10 μL at a flow rate of 0.8 mL min^{-1}, with (A) 0.1% (vol/vol) trifluoroacetic acid and (B) acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid as solvents. The following solvent gradients were then applied: 0–2.5 min, isocratic conditions, 10% B; 2.5–22 min, 10–49% B; 22–23 min, 49–10% B; 23–30 min, isocratic conditions, 10% B. The amount of para-κ-casein was quantified using LabSolutions Main (Shimadzu) software, based on the changes in the peak areas.

2.2.2. Measurement of protein coagulation

The coagulation process was carried out in a stress-controlled rheometer (MCR301 Anton Paar, Graz, Austria) equipped with a Couette geometry (Anton Paar; CC27, with a 28.93-mm cup diameter and a 26.64-mm bob diameter). Milk samples (20 mL) were equilibrated at each desired temperature for 15 min. After the addition of pepsin solution (200 μL), the samples were stirred for 30 s and loaded into the rheometer, which was set to the desired temperature. The C4 °C and W50 °C samples progressed through a temperature setting protocol. For the C4 °C sample, the temperature was gradually raised from 4 to 30 °C over the first 8 min, to 37 °C over 8–22 min and remaining at 37 °C to 120 min. For the W50 °C sample, the temperature was gradually decreased from 50 to 40 °C over the first 8 min, to 37 °C over 8–30 min and remaining at 37 °C to 120 min. A time sweep measurement was carried out at a constant frequency of 0.1 Hz with a strain of 1% (within the linear viscoelastic region as determined by strain sweep

measurements). The storage modulus (G') and the loss modulus (G'') were recorded every minute over 120 min.

2.2.3. Water-holding capacity of the milk curd

The water-holding capacity (WHC) of the samples was measured gravimetrically using a procedure from Yang et al. (2023). Milk (10 g) was placed in a 15-mL centrifuge tube and treated with the same concentration of pepsin (100 μL) at 25, 31, 37, or 43 °C. The samples were then centrifuged at 500 *g* for 2 min at room temperature. The expelled whey was carefully removed and weighed. WHC was calculated as the weight of the pellet expressed as a percentage of the weight of the total sample.

2.2.4. Microstructural characterization of the curd

The microstructure of the pepsin-induced curd at different temperatures was examined using CLSM. Fast Green fluorescent dye (3 μL; 1% wt/vol, in distilled water) was added to the skim milk (100 μL) and stirred for 30 s. After prewarming to 25, 31, 37, or 43 °C, pepsin was added to the milk, reaching a final pepsin concentration of 1.10 U (mL milk)$^{-1}$. The milk−pepsin mixture was then transferred to the cavity of a glass microscope slide and covered with a glass cover slip. The glass slides were incubated in an oven at each desired temperature for 120 min. The samples were then examined using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 63 × magnification lens at room temperature.

2.3. Dynamic gastric digestion

Dynamic gastric digestion was carried out using an HGS (Kong & Singh, 2010). The method described in Li, Ye, et al. (2022) was used in this study. Before the start of the gastric digestion, 20 mL of SGF (1 × concentrate, including pepsin) was added to the latex HGS chamber as the basal level in the fasted state. The oral phase of digestion was performed by mixing 20 g of SSF (prewarmed to 37 °C) into 200 g of milk (prewarmed to 4, 37, or 50 °C), equal to the solids content of the milk (Mulet-Cabero, Mackie, Brodkorb, & Wilde, 2020). The temperature of 4 °C and 50 °C samples after mixing was recorded. To simulate gastric secretion, a 1.25 × concentrated electrolyte SGF (pH 1.5) and the pepsin solution (pepsin and $CaCl_2$) were pumped gradually into the latex gastric chamber separately at flow rates of 2.0 and 0.5 mL min^{-1}, respectively. The HGS simulated the peristaltic movement in the stomach chamber at a frequency of three times per minute. The digestion was carried out for 240 min at a set temperature of 37 °C. The temperature of the gastric chyme was measured by a digital thermometer and recorded every 5 min. The digesta were emptied every 20 min (3 mL min^{-1}) through a 1-mm sieve to mimic gastric sieving and stored at −20 °C for further compositional analysis. The pH profiles during the digestion were determined every 20 min by measuring the freshly emptied digesta at 25 °C. The dilution curves of whey proteins in 60 g of digesta were calculated during the period of SGF secretion (including pepsin) and egress of the digesta.

The curds were sampled at 20, 60, and 240 min of digestion in triplicate for further analysis. Solid curds were collected by passing the contents of the gastric chamber through a 1-mm sieve. The wet weight of the fresh curds was determined immediately after sampling. A sub-sample of each curd sample was freeze dried and ground into a powder for further compositional analysis, and another sub-sample dried at 105 °C for 24 h. The dry weight and moisture content was measured by gravimetric analysis, based on the wet weight ratio between the whole curd and the portion taken for oven drying (Li, Pan, et al., 2022).

2.3.1. Macro- and microstructures of the digesta and the curds

To compare the macrostructures of the curds and the digesta, photographs of the fresh curds and the digesta samples were taken. CLSM imaging of the fresh curds was carried out as described in Ye, Cui, Dalgleish, and Singh (2017).

2.3.2. Rheological analysis of the curds

The rheological properties of the fresh curds were determined according to Li, Pan, et al. (2022). The complex moduli (G^*) of the milk curds at 20, 60, and 240 min were recorded after a 10-min time sweep with a shear strain of 0.5 and a frequency of 1 Hz at 37 °C, using a rheometer (AR-G2; TA Instruments, Crawley, West Sussex, UK) with a parallel plate geometry (40-mm diameter) and a 2 mm gap.

2.3.3. Texture analysis of the curds

The curd texture was measured as described by Roy, Ye, Moughan, and Singh (2021) using a TA-XT Plus texture analyzer (Stable Micro Systems, Surrey, UK), fitted with a 5.0-kg load cell and a 12.7-mm diameter cylinder probe. The curd was cut into 1.0 cm^3 cubes with a wire cutter immediately after sampling. Exponent software (version 6.1.15.0, Stable Micro Systems) was used for the analysis of results. The experiments were run, and the sample penetrated to a depth of 5.0 mm at a constant speed of 2.0 mm s^{-1}. The hardness value of the curds was calculated and expressed in Newtons.

2.3.4. Protein hydrolysis

The changes in the protein compositions of the curds and the emptied digesta were determined using RP-HPLC as described in Section 2.2.1. Sample buffer (0.8 mL) was added to 0.2 mL of digesta (immediately collected at 10, 20, 40, 60, 120, 180, and 240 min) or 8 mg of curd powder (collected at 20, 60, and 240 min and freeze dried). The protein content was determined by comparing the peak area of the proteins in the curds or digesta with that of the skim milk at each digestion time point.

2.4. Statistical analysis

Samples were prepared in triplicate from two different batches of milk, and the measurements of each sample were carried out in triplicate; values (or data points) are expressed as mean ± standard deviation. Experimental data were analyzed by running analysis of variance (ANOVA) tests using Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The hydrolysis kinetics and coagulation results were analyzed using one-way ANOVA with a Tukey post-hoc test. The changes in pH, wet and dry weights, moisture content, curd consistency, and protein hydrolysis were analyzed using two-way ANOVA, with the temperature of the ingested milk, the digestion time, and interaction (ingestion milk temperature × time) as fixed effects. If significant interaction effects were found, further analysis was carried out with Tukey post-hoc tests. Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Effect of temperature on the pepsin-induced hydrolysis of κ-casein and the coagulation of milk

3.1.1. Hydrolysis of κ-casein

Yang et al. (2022) reported that the pepsin-induced hydrolysis of κ-casein is pH dependent, and that the hydrolysis rate reaches a maximum at pH ~ 6.0 and 37 °C. Fig. 1A shows the degree of κ-casein hydrolyzed to para-κ-casein (%, written as degree of hydrolysis in the rest of the manuscript) at pH 6.0 after the addition of pepsin at different temperatures as a function of the reaction time. The experimental data were fitted to the equation:

$$ln\left(1-\frac{H_t}{100}\right)=\frac{K_{enz}\cdot C}{K_{den}}\cdot[exp(-K_{den}\cdot t)-1] \quad (1)$$

The reaction rate constants K ($K_{enz}\cdot$ C, min^{-1}) and K_{den} (min^{-1}) are shown in Table 1. Both K and K_{den} were significantly affected by temperature ($P < 0.05$). This indicates that the hydrolysis of κ-casein, as induced by pepsin, was markedly temperature-dependent. The

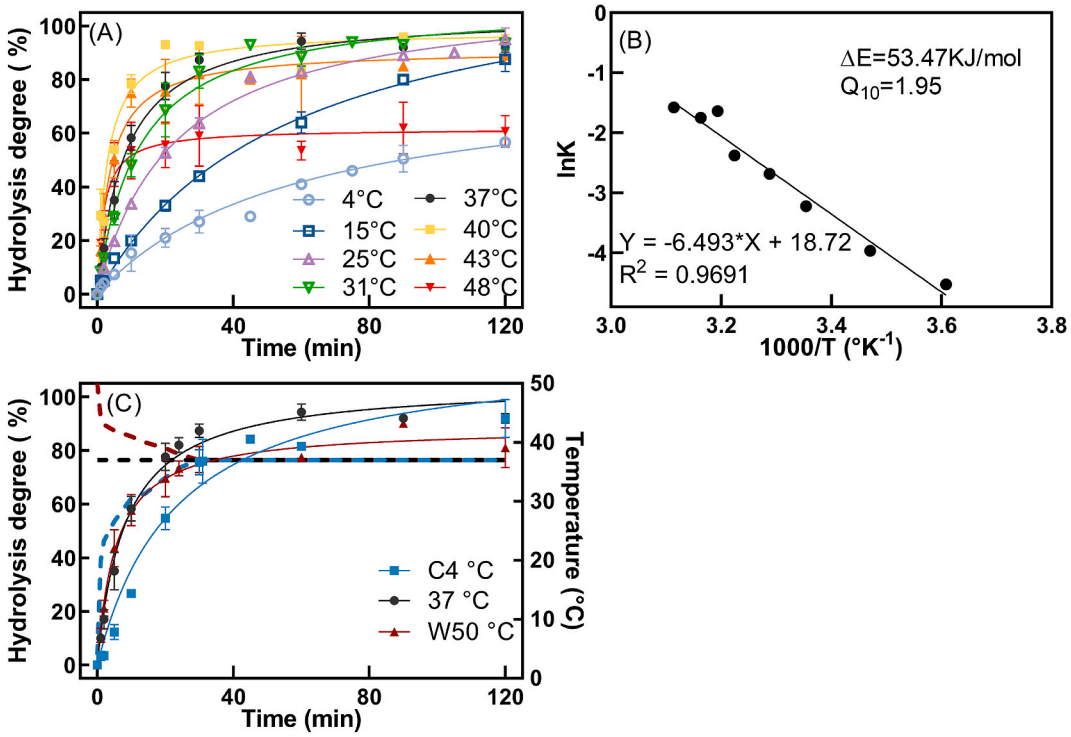


Fig. 1. (A) Degree of hydrolysis of κ-casein in skim milk as a function of time after the addition of 1.10 U pepsin mL^{-1} at different temperatures (○ 4 °C; □ 15 °C; △ 25 °C; ▽ 31 °C; ● 37 °C; ■ 40 °C; ▲ 43 °C; ▼48 °C). (B) Arrhenius plot of the reaction rate constant together with a least-squares linear fit through the experiment points. (C) Temperature profile of the C4 °C (▪▪▪), 37 °C (▪▪▪), and W50 °C (▪▪▪) milk samples during the measurement and the degree of hydrolysis of κ-casein in C4 °C (■), 37 °C (●), and W50 °C (▲) milk. Error bars represent standard deviations from triplicate measurements.

Table 1
Hydrolysis kinetics of κ-casein in skim milk at pH 6.0 with the addition of 1.1 U pepsin mL^{-1}.

Sample	Hydrolysis kinetics according to Eq. (1)	
	K (min^{-1})	K_{den}(min^{-1})
4 °C	0.0119 ± 0.0041 ^{e}	0.011 ± 0.009 ^{cd}
15 °C	0.0208 ± 0.0005 ^{e}	0.004 ± 0.004 ^{cd}
22 °C	0.0439 ± 0.0038 ^{de}	0.013 ± 0.005 ^{cd}
31 °C	0.0750 ± 0.0127 ^{cde}	0.023 ± 0.003 ^{cd}
37 °C	0.1021 ± 0.0187 ^{cd}	0.035 ± 0.008 ^{cd}
40 °C	0.2137 ± 0.0275 ^{ab}	0.081 ± 0.012 ^{bc}
43 °C	0.1910 ± 0.0384 ^{ab}	0.105 ± 0.007 ^{b}
48 °C	0.2272 ± 0.0533 ^{a}	0.267 ± 0.072 ^{a}
C4 °C	0.0432 ± 0.0013 ^{de}	0.007 ± 0.006 ^{d}
W50 °C	0.1257 ± 0.0101 ^{bc}	0.076 ± 0.022 ^{bc}

K, Enzymatic reaction rate constant (Kenz·C); K_{den}, Denaturation rate constant. $^{a-e}$ Mean values between samples in the same column with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

Arrhenius plot (Fig. 1B) shows the effect of temperature on the rate of the hydrolysis reaction. The Q_{10} for κ-casein hydrolysis induced by pepsin was calculated to be ~1.95, which is in the range of Q_{10} values for κ-casein hydrolysis by chymosin (1.3–2.0) (Van Hooydonk, 1987). Relatively high K values and low K_{den} values were found at 37–40 °C, indicating that the optimum temperature for pepsin to specifically hydrolyze κ-casein was around 37–40 °C. The highest K_{den} value was found at 48 °C, indicating that extensive pepsin denaturation occurred at this temperature. As a result, after 20 min, the degree of hydrolysis of κ-casein reached a plateau at around 60% (Fig. 1A), which was lower than for the samples at 31–43 °C.

The hydrolysis of the C4 °C and W50 °C milks was compared with that of the 37 °C milk (Fig. 1C). The dashed curves show the change in temperature of the C4 °C, 37 °C, and W50 °C milks, which increased or decreased to 37 °C over a period of 30 min. The W50 °C milk had significantly higher ($P < 0.05$) K and K_{den} values than the 37 °C and C4 °C milks. Therefore, the initial hydrolysis reaction rate between pepsin and κ-casein was faster in the W50 °C milk than in the 37 °C and C4 °C milks. Due to the more extensive denaturation of pepsin, the final degree of hydrolysis at 120 min was relatively lower for the W50 °C milk, at ~80%.

3.1.2. *Rheological properties*

The evolution of the storage modulus (G') as a function of time in the milk samples at various set temperatures (25, 31, 37, and 43 °C) is shown in Fig. 2A. Values for the coagulation time, the firming rate (dG'/dt), and G'_{max} are summarized according to Yang et al. (2022) in Table 2; all values were significantly affected by temperature ($P < 0.001$). At 25 °C, there was no significant increase in G' within 120 min (although it may have increased with a longer incubation time). The coagulation time decreased from ~39 to 13 min when the temperature was increased from 31 to 43 °C, in agreement with work of Panthi et al. (2019) on chymosin-induced coagulation. This probably occurred because of an increase in hydrophobic associations and enhanced enzymatic activity, which led to the formation of aggregates earlier in the coagulation process (Mishra, Govindasamy-Lucey, & Lucey, 2005).

The degree of hydrolysis at the coagulation time (H_{ct}) was calculated by substituting the coagulation time into the hydrolysis equation [Eq. (1)], and the results are shown in Table 2. Coagulation began before the end of the primary hydrolysis phase, when the degree of hydrolysis was ~83, 75, and 71% at 31, 37, and 43 °C, respectively. This indicates that relatively less degree of hydrolysis of κ-casein was required for

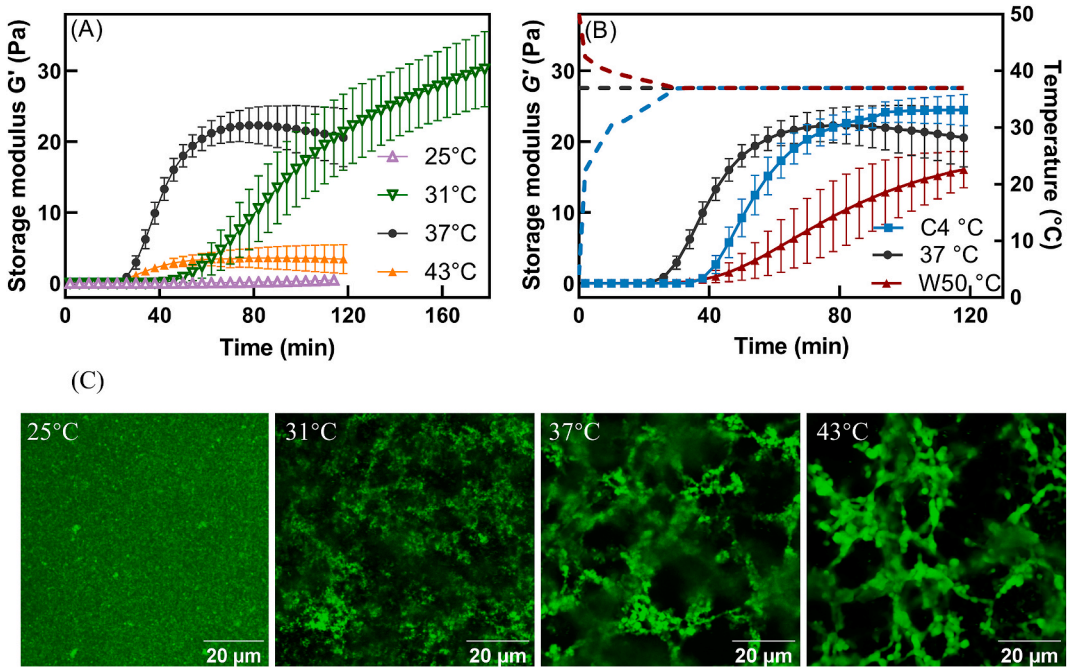


Fig. 2. (A) Storage modulus (G') of the pepsin-induced coagulum in samples at different constant set temperatures (△ 25 °C; ▽ 31 °C; ● 37 °C; ▲ 43 °C). (B) G' of the pepsin-induced coagulum in C4 °C (▪), 37 °C (●), and W50 °C (▲) milk at pH 6.0 and temperature profile of the C4 °C (▪▪▪), 37 °C (▪▪▪), and W50 °C (▪▪▪) milk samples during the associated measurements. Error bars represent standard deviations from triplicate measurements. (C) Microstructures of the pepsin-induced coagulum at 120 min at different constant set temperatures (25, 31, 37, 43 °C) as analyzed by confocal laser scanning microscopy. The scale bars are 20 μm in length for all micrographs.

Table 2
Effect of temperature on the coagulation properties of skim milk at pH 6.0 with the addition of 1.1 U pepsin mL^{-1}.

	25 °C	31 °C	37 °C	43 °C	C4 °C	W50 °C
Coagulation time (min)	>120	39.48 ± 5.17 ^{b}	20.80 ± 1.08 ^{c}	13.05 ± 2.14 ^{d}	33.43 ± 2.64 ^{b}	25.26 ± 2.96 ^{c}
Firming rate (Pa min^{-1})	–	0.41 ± 0.06 ^{b}	0.95 ± 0.06 ^{a}	0.13 ± 0.03 ^{d}	0.91 ± 0.12 ^{c}	0.34 ± 0.05 ^{b}
G'_{max} (Pa)	–	28.60 ± 1.91 ^{a}	22.57 ± 2.75 ^{b}	4.04 ± 1.09 ^{d}	23.93 ± 1.81 ^{ab}	16.33 ± 2.27 ^{c}
H_{ct} (%)	–	83 ± 2 ^{b}	75 ± 1 ^{c}	71 ± 3 ^{cd}	69 ± 3 ^{d}	74 ± 2 ^{cd}
WHC (%)	–	58.46 ± 9.46 ^{a}	19.64 ± 2.25 ^{b}	12.11 ± 1.28 ^{c}	–	–

G'_{max}, maximum storage modulus value during the measuring time; H_{ct}, degree of hydrolysis of κ-casein at the coagulation time; WHC, water-holding capacity. $^{a-d}$ Mean values between samples in the same row with different superscripts are significantly different ($P < 0.05$). The results are expressed as the mean ± the standard deviation of the mean ($n = 3$).

coagulation to occur at higher temperatures, in agreement with Carlson, Hill, and Olson (1987) and Garg and Johri (1994). Greater hydrophobic association and diffusion (due to Brownian motion) between casein molecules is expected at higher temperatures (Dalgleish, 1983; Horne & Lucey, 2014; O'meara & Munro, 1982). Since the increasing number of hydrophobic domains in casein, generated from the hydrolysis process, seek to reduce contact with water molecules, a lowering of energy favors self-association; an increased frequency of collisions between the para-casein micelles would facilitate coagulation at a lower degree of κ-casein hydrolysis.

The maximum slope of the G' curve (dG'/dt) is defined as the firming rate and was significantly higher ($P < 0.05$) at 37 °C than at 25, 31, and 43 °C (Table 2). Fig. 2A shows that the G' of the sample at 37 °C was higher than that of the sample at 31 °C until ~115 min. However, beyond 115 min, the G' of the sample at 31 °C increased continually whereas that of the sample at 37 °C reached a plateau (decreasing slightly); thus, a higher G' was found in the later stages at 31 °C. Therefore, as shown in Table 2, G'_{max} decreased with increasing temperature. This is consistent with Mellema et al. (2002), who reported that a higher G' of chymosin-induced curd was found at a higher temperature (25 and 30 °C were compared), as long as the curd was not fully developed (*i.e.*, in the early stage of coagulation). As mentioned above, stronger hydrophobic association and increased frequency of collisions between the para-casein micelles perhaps resulted in more rapid intra-/interparticle protein rearrangements (types A and B according to Mellema et al. (2002)). This led to the formation of a stronger protein network at higher temperatures up to 37 °C, as indicated by the faster G' development(Sandra, Cooper, Alexander, & Corredig, 2011). In the later stages of coagulation, the lower G' at 37 °C could have been due to the more extensive inter-cluster rearrangements of the curd at 37 °C, resulting in syneresis, as indicated by a decrease in G' after 80 min. For the sample at 43 °C, G' was lower than that for the sample at 37 °C. There is little published information on the rheological properties of enzyme-induced coagulated milk at temperatures >40 °C. However, as the fusion of para-casein micelles is expected to increase rapidly at higher temperatures because of greater hydrophobic association, faster syneresis would occur concurrently; the evolution of G' could therefore be related to the fact that the increase in firmness of the coagulum was masked by syneresis (Lagoueyte, Lagaude, & De La Fuente, 1995). In addition, according to Zoon, Van Vliet, and Walstra (1988), G' was lower at 40 °C than at 35 °C, probably because of the inactivation of chymosin at the higher temperature. In the present study, the rate of hydrolysis at 43 °C was rapid during the first 10 min (Fig. 1A); however, the degree of hydrolysis reached a plateau at ~80% due to denaturation of pepsin. Fewer exposed para-casein surfaces in the curd network could also attribute the lower firming rate and lower G'_{max}.

The evolution of G' for the C4 °C and W50 °C milks was compared with that of the 37 °C milk in Fig. 2B and the rheological parameters are

summarized in Table 2. The C4 °C and W50 °C milks coagulated at ~33 and 25 min, respectively, when ~70% of the caseins had been hydrolyzed. The coagulation time was longer for the C4 °C milk than for the 37 °C and W50 °C milks. Dissociation of β-casein has been reported in 4 °C milk, which could generate more space internally in the casein micelle (Zhang et al., 2018), and resulted in the longer coagulation time. On the other hand, it may have been related to the hydrolysis kinetics, in that, during the first 40 min, the degree of hydrolysis of κ-casein in the C4 °C milk was relatively lower than that in the other two milks (Fig. 1C). As the aggregation was markedly dependent on the temperature, the lower temperature resulted in slower aggregation. The firming rate and G'_{max} of C4 °C milk were ~0.91 Pa min^{-1} and 23.93 Pa, respectively, which were similar to those of the 37 °C milk. However, the firming rate and G'_{max} for the W50 °C milk were 0.34 Pa min^{-1} and 16.33 Pa, respectively, i.e., lower than for the other two milks. As the hydrolysis reaction rate constant K_{enz} and the denaturation rate constant K_{den} were similar for the C4 °C and the 37 °C milk ($P > 0.05$), the developments of G' were similar. The relatively lower firming rate and G'_{max} for the W50 °C milk can be attributed to the hydrolysis kinetics. Even though the temperature decreased to 37 °C after around 30 min, the denaturation of the pepsin was relatively high for the W50 °C milk. The degree of hydrolysis of the casein micelles was maintained at ~80% from 40 to 120 min, lower than for the C4 °C and 37 °C milk samples [Fig. 1C]. As a consequence, fewer exposed para-casein surfaces were involved in curd formation, resulting in the lower G'_{max}.

3.1.3. Water-holding capacity and microstructure of the curd

As shown in Table 2, the WHCs of the curds were significantly influenced by temperature, *i.e.*, highest at 31 °C, followed by 37 and 43 °C. In agreement with Teo, Munro, Singh, and Hudson (1996), the lower WHC could be largely attributed to increased hydrophobic association between the casein molecules with an increase in temperature. In addition, the difference in WHCs of the curds could also be related to the network structures formed during coagulation. As shown in Fig. 2C, temperature had a significant effect on the curd microstructure. Except for the sample at 25 °C, which did not show a network structure the casein micelles assembled into strands and were interconnected by large numbers of crosslinks in the samples at 31, 37, and 43 °C. A lower level of crosslinks with larger strands was observed in the samples at higher temperature. This is consistent with the findings of Ong, Dagastine, Auty, Kentish, and Gras (2011), who observed that chymosin-induced curds formed a finer network when the milk was treated at 27 °C compared with 36 °C. At 43 °C, the casein aggregates and the pores in the network were large. This may have been due to the rapid formation of larger aggregates at temperatures >40 °C (Muñoz, Torres, Guerrero, & Talavera, 2017) and extensive inter-cluster rearrangements (Mellema et al., 2002). Under an external centrifugation force (500 *g*), the larger pores in the samples at higher temperatures may have been more easily

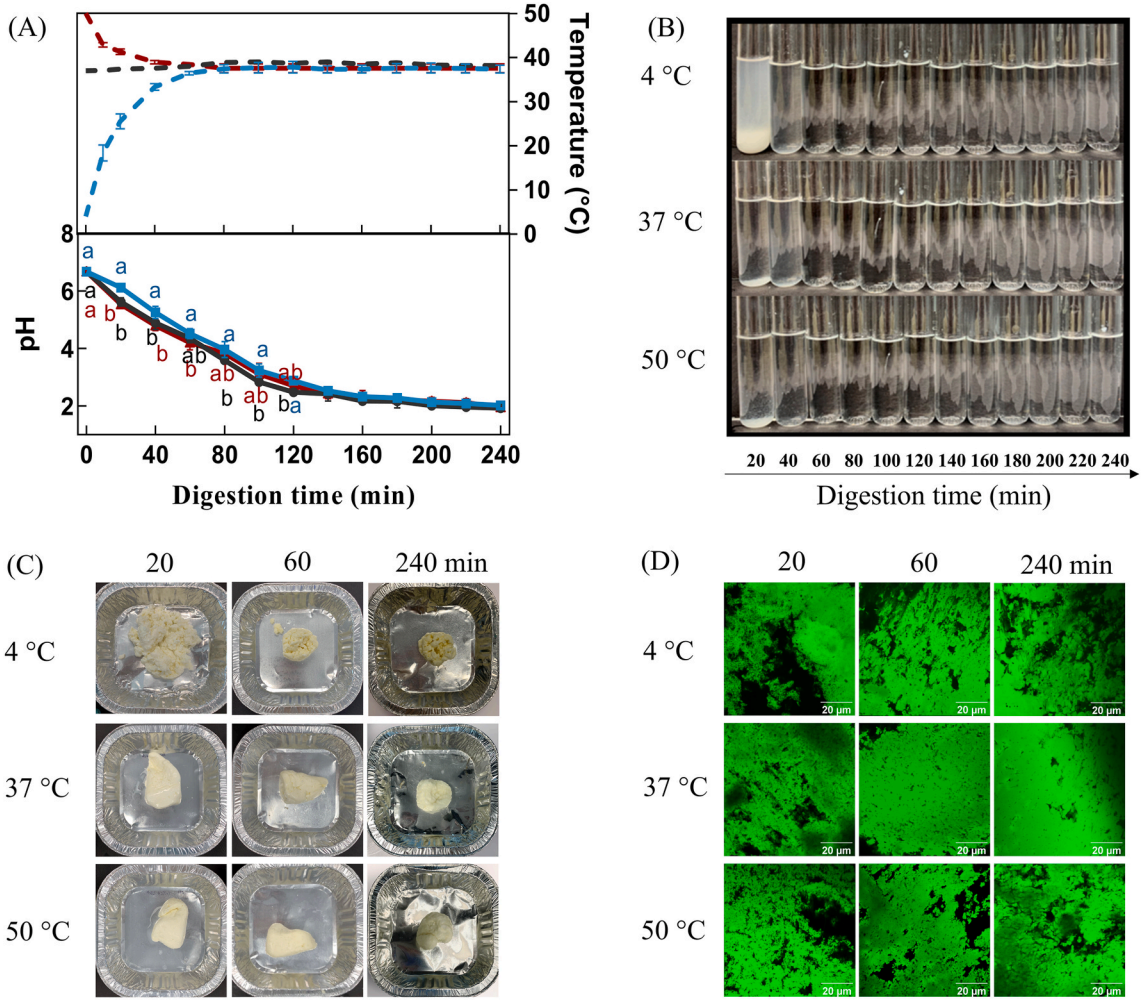


Fig. 3. (A) Measured pH (solid curves with symbols) and temperature (dashed curves) profiles of the gastric digesta during 240 min of digestion. Blue color: 4 °C milk; black color: 37 °C milk; Red color: 50 °C milk. $^{a-c}$ Mean values between samples with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements. (B) Photographs of emptied digesta from 20 to 240 min. (C) Photographs (macrostructure) and (D) confocal micrographs (microstructure) of the coagulum formed following 20, 60, and 240 min of gastric digestion in the human gastric simulator.

destroyed, with water (liquid) being expelled, resulting in a lower WHC.

3.2. In vitro digestion behavior of 4 °C, 37 °C and 50 °C milks

3.2.1. Temperature and pH changes during digestion

The temperature of the HGS was set at 37 °C (body temperature). The temperature profiles of the milks during digestion are shown in Fig. 3A. The 4 °C milk at 4 °C gradually increased to 37 °C over 60 min; the 50 °C milk at 50 °C decreased to 37 °C over 60 min. The digesta were collected every 20 min and the pH was measured at 25 °C. The pH values decreased from ~6.7 to ~2.0 over 240 min (Fig. 3A) and were significantly affected by the digestion time and the temperature of the ingested milk ($P < 0.001$). The pH of the 4 °C milk decreased more slowly than those of the 37 °C and 50 °C milks during the first 40 min (0.2–0.3 units higher), indicating a slightly higher buffering capacity of the milk at the lower temperature. From 40 to 240 min, the pH profile became similar for all three samples.

3.2.2. Macro-/microstructures of the curd and digesta during digestion

Gastric coagulation of all milk samples occurred at pH > 6.0, in agreement with Mulet-Cabero, Mackie, Wilde, Fenelon, and Brodkorb (2019) and Ye et al. (2017). During the experiments, protein coagulation was visible immediately after the addition of 37 °C and 50 °C milk into HGS, whereas visible coagulation occurred at a later time for the 4 °C milk. The late coagulation of the 4 °C milk was consistent with the observation that C4 °C milk had a longer coagulation time than the 37 °C and 50 °C milks at pH 6.0 (Table 2). The appearances of the digesta are shown in Fig. 3B. At 20 min, the digesta of 4 °C milk was more turbid than the 37 °C and 50 °C milks. This explains the relatively higher pH of the 4 °C milk during the first 40 min which arises from the buffering capacity of colloidal calcium phosphate and milk proteins in the liquid phase. In general, the digesta became clearer with an increase in the digestion time, and lower turbidity could be observed after 240 min of digestion.

Photographs of the curds formed during gastric digestion of the 4 °C, 37 °C and 50 °C milks at 20, 60 and 240 min are presented in Fig. 3C. After 20 min of digestion, the curds formed from the 37 °C and 50 °C milks were intact with fairly smooth surfaces, whereas the curd formed from the 4 °C milk was crumbly and fragmented with small granules. After further digestion, at 60 min, the 4 °C milk curd was firmer with a smooth surface. At this stage, the serum became clear indicating that most of the casein was incorporated into the curd. At 240 min, the entire block of the curd was smaller but remained integrated. These results indicate that the initial temperature of the milk could lead to the formation of differently structured curds during the early stage of digestion.

CLSM images of the curd samples at 20, 60, and 240 min are given in Fig. 3D and reveal the protein microstructure of the curds. These images are consistent with curd formation in the HGS that was observed visually (macrostructure, Fig. 3C). At 20 min, the curd network consisted of a loose, irregular protein matrix with tiny pores. After 240 min of digestion, there were fewer pores in all samples, which may have been due to contraction and exchange of water between the curd and the gastric fluid during digestion. Liquid in the pores was expelled with mechanical deformation of the curd. The protein matrix for the 4 °C curd sample appeared to be loose, with an open and porous structure at all times during digestion. The microstructure of the 37 °C curd sample was denser than those of the other two samples at all time points.

3.2.3. Curd weights and moisture

The dry matter content, water content, and wet weight (total content) of the curds at 20, 60, and 240 min are presented in Fig. 4A. The wet weight and the water content were significantly affected by the digestion time and by the interaction between the digestion time and the temperature of ingested milk ($P < 0.001$). The wet weight of the curds followed the order 4 °C > 37 °C ≈ 50 °C at the beginning of the digestion (20 min) and the order 4 °C < 37 °C ≈ 50 °C after 60 min of digestion.

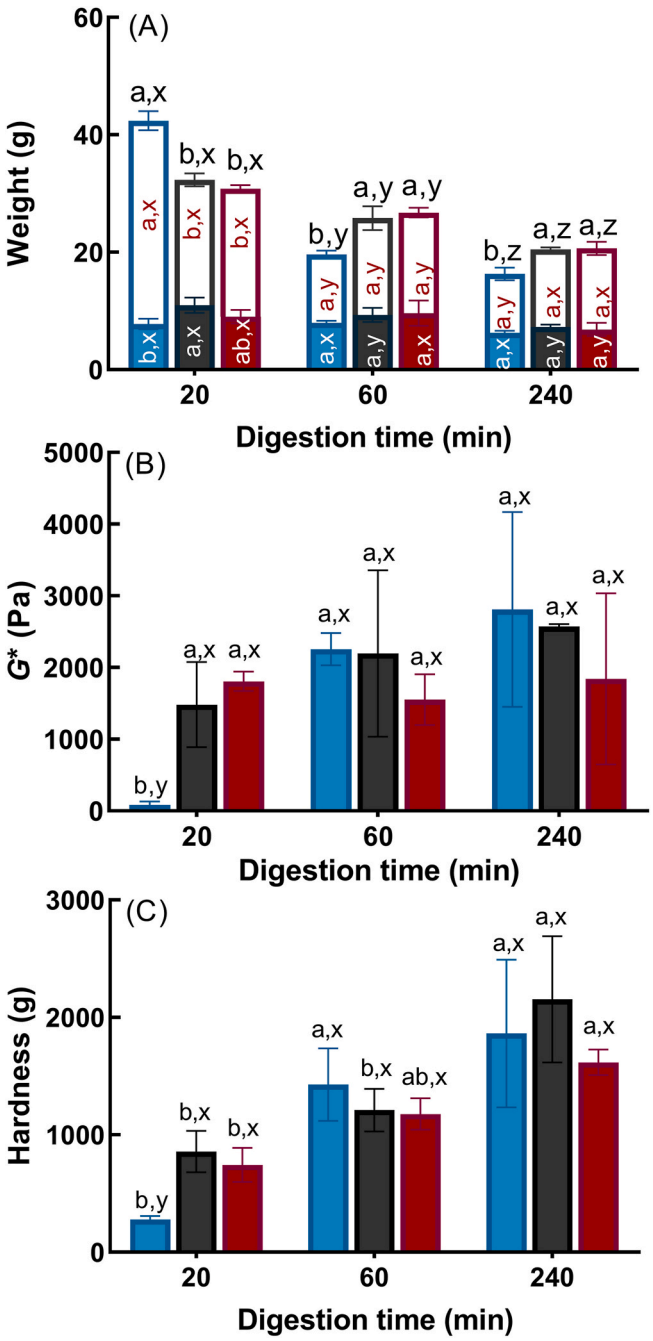


Fig. 4. (A) Dry matter weight (bottom) and water weight (top) of fresh clots formed during the gastric digestion at 20, 60, and 240 min. (B) G^* values of the coagulum formed during the gastric digestion at 20, 60, and 240 min. (C) Hardness of the coagulum formed during the gastric digestion at 20, 60, and 240 min. Blue color: 4 °C milk; black color: 37 °C milk; Red color: 50 °C milk. $^{x-z}$ Mean values for the same sample at different digestion times with different superscripts are significantly different ($P < 0.05$). $^{a-b}$ Mean values between samples at the same digestion time with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements.

The wet weights of all curds decreased significantly over the period of digestion. This occurred because more proteins in the curd were hydrolyzed into peptides or amino acids during digestion (discussed in Section 3.2.5); thus, the dry matter content decreased with an increase in digestion time. In addition, water was expelled with mechanical deformation of the curds by peristaltic movement in the HGS. At each digestion time, the wet weights of the curds formed from the 37 °C and

50 °C milks did not vary significantly (P > 0.05), whereas the wet weight of the curd formed from the 4 °C milk was relatively higher (P < 0.05) at 20 min. This can be explained by the structure of the protein matrix during the early stages of digestion. It is expected that fragmented curds would have larger surface areas which would bind more water (Li, Pan, et al., 2022). As mentioned previously, the curd structure for the 4 °C milk was relatively looser and a greater number of small particles passed into the liquid phase, resulting in the highest water content and the lowest dry matter content at 20 min. In the later stages (60 and 240 min), the water content in the 4 °C milk curd decreased significantly and became similar to those of the other two curds. The water contents of the curds formed from the 37 °C and 50 °C milks were similar and remained constant throughout the whole digestion process. In summary, the curd weights of the 37 °C and 50 °C milks were similar at each digestion time, whereas a lower total weight was found in the curd formed from the 4 °C milk after 240 min of digestion.

3.2.4. Rheological and texture analysis of the curds

Fig. 4B presents the G^* values for the curds formed by the 4 °C, 37 °C and 50 °C milks at different digestion times. The G^* value can be used as an indicator of curd consistency (Li, Pan, et al., 2022; Mulet-Cabero et al., 2019). The G^* values of the curds formed from the 37 °C and 50 °C milks did not change markedly during digestion ($P > 0.05$), at around 2000 Pa. However, the G^* value of the curd formed from the 4 °C milk was ~84 Pa in the early stages of digestion (20 min), which was significantly lower ($P < 0.05$) than those of the curds formed from the 37 °C and 50 °C milks. The G^* value of the curd formed from the 4 °C milk increased significantly and became similar to those of the curds formed from the 37 °C and 50 °C milks with an increase in digestion time (at 60 and 240 min).

According to Roy et al. (2021), texture analysis gives an indication of the heterogeneous and dynamic nature of the curds during gastric digestion. The hardness of the curds formed by the 4 °C, 37 °C and 50 °C milks at different digestion times are shown in Fig. 4C. A significantly

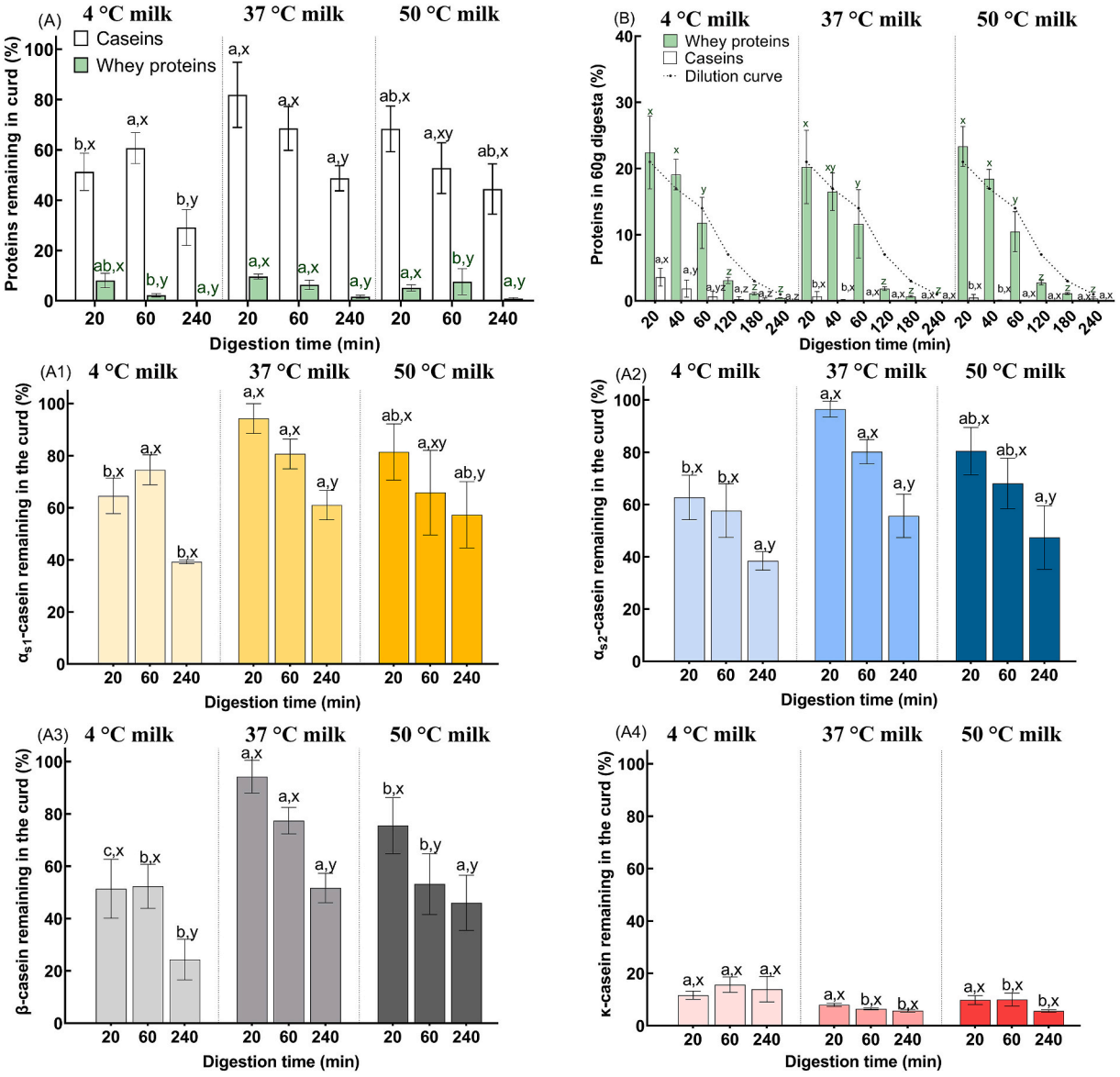


Fig. 5. (A) Percentage of caseins (□) and whey proteins (■) remaining in the clot at 20, 60, and 240 min (A1)–(A4) Percentage of αs_1-casein, αs_2-casein, β-casein, and κ-casein remaining in the clot at 20, 60, and 240 min. (B) Percentage of caseins (□) and whey proteins (■) detected in the 60 g of emptied digesta at 20, 40, 60, 120, 180, and 240 min. The dashed curves are the expected trends of the whey protein content resulting from the dilution caused by the SGF addition. $^{x-z}$ Mean values for the same sample at different digestion times with different superscripts are significantly different ($P < 0.05$). $^{a-b}$ Mean values between samples at the same digestion time with different superscripts are significantly different ($P < 0.05$). Error bars represent standard deviations from triplicate measurements.

lower hardness ($P < 0.05$) was found in the curd formed from the 4 °C milk at 20 min. However, no significant differences ($P > 0.05$) were detectable for hardness at 240 min for all samples.

During digestion, a structural rearrangement of the protein network may have taken place due to mechanical peristaltic movement and more water may have been expelled from the curd over time. According to Li, Pan, et al. (2022) and Roy et al. (2021), the consistency and the texture of the curd are negatively correlated with moisture content. Therefore, this contributed to the formation of a stronger curd (higher values of G^* and hardness) in the HGS during the later stages of dynamic gastric digestion. The lowest consistency and hardness were found in the 4 °C milk curd at 20 min. This could be attributed to the more open structure (Fig. 5B and C) and the higher moisture content (Fig. 4A) that were observed in the 4 °C milk curd at 20 min.

3.2.5. Protein content in the curds and digesta during digestion

The changes in specific protein components in the curds and the digesta during digestion, as determined by RP-HPLC, are shown in Supplemental Figs. 1 and 2, respectively. Based on the peak area, the amount of each major protein (αs_1-casein, αs_2-casein, β-casein, κ-casein, whey proteins) was calculated. The percentage of each major protein remaining in the curd or digesta was calculated as the ratio of each protein remaining in the curd or digesta to each protein in the 200 mL of milk that was ingested in the HGS (wt/wt). Fig. 5A shows the percentages of casein and whey proteins remaining in the curd, and Fig. 5B shows the percentages remaining in the digesta. The hydrolysis of casein proteins in the curds and digesta was significantly affected by the digestion time ($P < 0.05$) and by the temperature of the ingested milk ($P < 0.05$). The hydrolysis of the whey proteins in the curds and digesta was significantly affected by the digestion time ($P < 0.05$) but was not significantly affected by the temperature of the ingested milk ($P > 0.05$). In agreement with previous research, most of the whey proteins in unheated samples are soluble and present in the liquid digesta (Ye et al., 2016). The small amount of whey proteins observed in the curds may have been caused by entrapment during the formation of the curds; these could have been expelled from the curds and gradually hydrolyzed as digestion progressed.

The percentages of each individual casein protein remaining in the curd during digestion are shown in Fig. 7A1–A4. At 20 min, little of the intact κ-casein (12, 8, and 10% for the 4 °C, 37 °C, and 50 °C milks, respectively) remained in the curds of all milk samples. Because of the rapid specific hydrolysis at pH > 6, κ-casein was expected to be hydrolyzed to para-κ-casein within the first several minutes. A decrease in intact proteins and an increase in peptides was observed after 240 min of digestion for all samples (Supplemental Figs. 1 and 2). Due to the slower coagulation and looser structure (Fig. 3C) of the 4 °C milk sample, some of the casein proteins and/or some small crumbly particles (containing mostly caseins) were still in the liquid phase (Fig. 5B). From 20 to 60 min of digestion, these casein proteins gradually assembled into the curds, which resulted in the relatively higher average value of caseins (especially αs_1-casein, Fig. 5A1) for the 4 °C sample in Fig. 5A. After 240 min of digestion, the lowest percentages of caseins were found in the 4 °C milk curd, whereas there was no significant difference ($P > 0.05$) between the 37 °C and 50 °C milk curds (Fig. 5A). This indicated that 4 °C milk led to a faster release rate of the casein proteins during gastric digestion. Faster degradation of proteins has been shown to be caused by the looser and crumbly structure of curds at the early stage of digestion, which allows pepsin to diffuse into the curds rapidly and to hydrolyze the proteins (Ye et al., 2017). There was no significant difference between the amount of each individual protein as a fraction of total protein in the curds (results not shown) during the whole period of digestion, indicating that the hydrolysis rate of each protein type by pepsin was similar in each curd.

A small amount of casein protein was present at 20 min in the digesta for all samples (Fig. 5B), likely due to some caseins not coagulated and incorporated into the curd, and were therefore emptied from the stomach into the digesta. As discussed above, because of the looser structure of the curd in the 4 °C milk sample, a greater amount of casein proteins ($P < 0.05$) was found in the 4 °C milk sample digesta at 20 min. The values shown as grey dashed lines in Fig. 5B are expected trends of whey proteins based on dilution due to the continual addition of SGF. After 120 min, the amount of whey proteins decreased to below the dilution (dashed line) caused by the continuous addition of SGF. This may have been because of the hydrolysis of α-lactalbumin by pepsin at pH < 4 (Wang, Ye, Lin, Han, & Singh, 2018).

These results suggested that the dry matter in the curds was mainly casein proteins for all milk samples. The protein emptied from the stomach was composed mainly of whey proteins in the early stages of digestion and was digested to peptides by pepsin at longer digestion times. After 240 min of digestion, fewer intact proteins were found in the 4 °C milk curd, indicating a faster rate of hydrolysis.

4. Conclusions

The present study demonstrates the impact of temperature on pepsin-induced hydrolysis and coagulation of milk proteins. The temperature coefficient of the hydrolysis of κ-casein was ~1.95. The optimum temperature for pepsin to specifically hydrolyze κ-casein was found to be around 37–40 °C. The coagulation behavior and the structure of the curd depended markedly on the set temperature. Different coagulation and digestion behaviors were found for the milks with different initial temperatures during dynamic *in vitro* gastric digestion. It took longer to coagulate under the gastric conditions for the 4 °C milk than for the 50 °C milk or the milk at body temperature. Protein digestion was relatively faster for the 4 °C milk than for the other two milk samples, which was attributed to the looser and softer structure during the early stages of digestion. After 240 min of digestion, the total curd weights of the 37 °C and 50 °C milk samples were similar but higher than that of the 4 °C milk sample. The findings of the present study provide insight into gastric coagulation and digestion of milk at different initial temperatures.

CRediT authorship contribution statement

Mengxiao Yang: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **Aiqian Ye:** Conceptualization, Funding acquisition, Supervision, Resources, Writing – review & editing. **Zhi Yang:** Supervision, Writing – review & editing. **David W. Everett:** Supervision, Writing – review & editing. **Elliot Paul Gilbert:** Supervision, Writing – review & editing. **Harjinder Singh:** Supervision, Resources, Writing – review & editing.

Declaration of competing interest

There are no conflicts of interest to declare.

Data availability

Data will be made available on request.

Acknowledgments

This study was funded by the New Zealand Milks Mean More (NZ3M) program and the Riddet Institute Centre of Research Excellence, Tertiary Education Commission, New Zealand. The author Mengxiao Yang thanks the Australian Institute of Nuclear Science and Engineering (AINSE) for a Post Graduate Research Award. We would like to acknowledge the support of Michael Agnew (AgResearch Limited, New Zealand) for helping with the RP-phase HPLC and Claire Woodhall (Havelock North, New Zealand) for proofreading the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2023.108550.

References

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*(4), 991–1014.

Carlson, A., Hill, C. G., Jr., & Olson, N. F. (1987). Kinetics of milk coagulation: III. Mathematical modeling of the kinetics of curd formation following enzymatic hydrolysis of κ-casein—parameter estimation. *Biotechnology and Bioengineering, 29*(5), 601–611.

Dalgleish, D. G. (1983). Coagulation of renneted bovine casein micelles: Dependence on temperature, calcium ion concentration and ionic strength. *Journal of Dairy Research, 50*(3), 331–340.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. (2017). *Enzymatic coagulation of milk. In Fundamentals of cheese science*. Boston: Springer.

Garg, S. K., & Johri, B. N. (1994). Rennet: Current trends and future research. *Food Reviews International, 10*(3), 313–355.

Gunasekaran, S., & Ay, C. (1996). Milk coagulation cut-time determination using ultrasonics. *Journal of Food Process Engineering, 19*(1), 63–73.

Horne, D., & Lucey, J. (2014). Revisiting the temperature dependence of the coagulation of renneted bovine casein micelles. *Food Hydrocolloids, 42*, 75–80.

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

Kong, F., & Singh, R. P. (2010). A human gastric simulator (HGS) to study food digestion in human stomach. *Journal of Food Science, 75*(9), E627–E635.

Lagoueyte, N., Lagaude, A., & De La Fuente, B. T. (1995). Rheological properties of renneted reconstituted milk gels by piezoelectric viscoprocess: Effects of temperature and calcium phosphate. *Journal of Food Science, 60*(6), 1344–1348.

Li, S., Pan, Z., Ye, A., 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.

Li, S., Ye, A., Pan, Z., Cui, J., Dave, A., & Singh, H. (2022). Dynamic in vitro gastric digestion behavior of goat milk: Effects of homogenization and heat treatments. *Journal of Dairy Science, 105*(2), 965–980.

Mellema, M., Walstra, P., Van Opheusden, J., & Van Vliet, T. (2002). Effects of structural rearrangements on the rheology of rennet-induced casein particle gels. *Advances in Colloid and Interface Science, 98*(1), 25–50.

Mishra, R., Govindasamy-Lucey, S., & Lucey, J. A. (2005). Rheological properties of rennet-induced gels during the coagulation and cutting process: Impact of processing conditions. *Journal of Texture Studies, 36*(2), 190–212.

Mulet-Cabero, A.-I., Mackie, A. R., Brodkorb, A., & Wilde, P. J. (2020). Dairy structures and physiological responses: A matter of gastric digestion. *Critical Reviews in Food Science and Nutrition*, 1–16.

Mulet-Cabero, A.-I., Mackie, A. R., Wilde, P. J., Fenelon, M. A., & Brodkorb, A. (2019). Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk. *Food Hydrocolloids, 86*, 172–183.

Muñoz, S. V., Torres, M. G., Guerrero, F. Q., & Talavera, R. R. (2017). A new study of the kinetics of curd production in the process of cheese manufacture. *Journal of Dairy Research, 84*(4), 479–483.

Nájera, A., De Renobales, M., & Barron, L. (2003). Effects of pH, temperature, CaCl2 and enzyme concentrations on the rennet-clotting properties of milk: A multifactorial study. *Food Chemistry, 80*(3), 345–352.

O'meara, G., & Munro, P. (1982). The precipitation and shrinkage of acid casein curd: A preliminary study. *New Zealand Journal of Dairy Science & Technology, 17*, 147–159.

Ong, L., Dagastine, R. R., Auty, M. A., Kentish, S. E., & Gras, S. L. (2011a). Coagulation temperature affects the microstructure and composition of full fat Cheddar cheese. *Dairy Science & Technology, 91*(6), 739–758.

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2011b). Microstructure of milk gel and cheese curd observed using cryo scanning electron microscopy and confocal microscopy. *LWT–Food Science and Technology, 44*(5), 1291–1302.

Panthi, R. R., Kelly, A. L., Sheehan, J. J., Bulbul, K., Vollmer, A. H., & McMahon, D. J. (2019). Influence of protein concentration and coagulation temperature on rennet-induced gelation characteristics and curd microstructure. *Journal of Dairy Science, 102*(1), 177–189.

Roy, D., Ye, A., Moughan, P. J., & Singh, H. (2021). Structural changes in cow, goat, and sheep skim milk during dynamic in vitro gastric digestion. *Journal of Dairy Science, 104*(2), 1394–1411.

Sandra, S., Cooper, C., Alexander, M., & Corredig, M. (2011). Coagulation properties of ultrafiltered milk retentates measured using rheology and diffusing wave spectroscopy. *Food Research International, 44*(4), 951–956.

Sun, W., Houghton, L., Read, N., Grundy, D., & Johnson, A. (1988). Effect of meal temperature on gastric emptying of liquids in man. *Gut, 29*(3), 302–305.

Teo, C. T., Munro, P. A., Singh, H., & Hudson, R. C. (1996). Effects of pH and temperature on the water-holding capacity of casein curds and whey protein gels. *Journal of Dairy Research, 63*(1), 83–95.

Turhan, M., & Mutlu, M. (1998). Kinetics of κ-casein/immobilized chymosin hydrolysis. *Enzyme and Microbial Technology, 22*(5), 342–347.

Van Hooydonk, A. (1987). *The renneting of milk: A kinetic study of the enzymic and aggregation reactions*. Van Hooydonk.

Wang, X., Ye, A., Lin, Q., Han, J., & Singh, H. (2018). Gastric digestion of milk protein ingredients: Study using an in vitro dynamic model. *Journal of Dairy Science, 101*(8), 6842–6852.

Webber, D., Nouri, M., & Bell, F. (1980). A study of the effects of meal temperature on gastric function. *Pflügers Archiv, 384*(1), 65–68.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2022). Kinetics of pepsin-induced hydrolysis and the coagulation of milk proteins. *Journal of Dairy Science, 105*(2), 990–1003.

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Pepsin-induced coagulation of casein micelles: Effect of whey proteins and heat treatment. *Food Chemistry, 402*, Article 134214.

Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2016). Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis. *Food Hydrocolloids, 52*, 478–486.

Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2017). Effect of homogenization and heat treatment on the behavior of protein and fat globules during gastric digestion of milk. *Journal of Dairy Science, 100*(1), 36–47.

Ye, A., Liu, W., Cui, J., Kong, X., Roy, D., Kong, 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, Y., Liu, D., Liu, X., Huang, F., Zhou, P., Zhao, J., et al. (2018). Effect of temperature on casein micelle composition and gelation of bovine milk. *International Dairy Journal, 78*, 20–27.

Zhao, L., Budge, S. M., Ghaly, A. E., Brooks, M. S., & Dave, D. (2011). Extraction, purification and characterization of fish pepsin: A critical review. *Journal of Food Processing & Technology, 2*(6), 1.

Zoon, P., Van Vliet, T., & Walstra, P. (1988). Rheological properties of rennet-induced skim milk gels. 2. The effect of temperature. *Netherlands Milk and Dairy Journal, 42*(3), 271–294.