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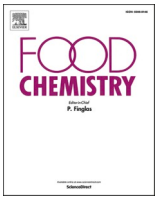

Gastric clot formation and digestion of milk proteins in static *in vitro* infant gastric digestion models representing different ages

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ABSTRACT

Gastric digestion conditions change during infancy from newborn towards more adult digestion conditions, which can change gastric digestion kinetics. However, how these changes in gastric digestion conditions during infancy affect milk protein digestion has not been investigated. Therefore, we aimed to investigate milk protein digestion with static *in vitro* gastric digestion models representing one-, three- and six-month-old infants. With increasing age, gastric clots and soluble proteins were digested more extensively, which may partly be attributed to the looser gastric clot structure. Larger differences with increasing age were found for heated than unheated milk proteins, which might be caused by the presence of denatured whey proteins. Taken together, these findings show that gastric milk protein digestion increases during infancy. These *in vitro* gastric digestion models could be used to study how milk protein digestion changes with infant age, which may aid in developing infant formulas for different age stages.

1. Introduction

Mother's milk is considered the best nutrition for infants, and drinking mother's milk is associated with several health benefits, including a reduced occurrence of respiratory and gastrointestinal infectious diseases (Brodkorb et al., 2019). These mother's milk-related health benefits may partially be explained by the differences in composition of mother's milk during lactation, which were shown to coincide with the development of the digestive and immune system of infants (Zhang et al., 2016). In contrast, no different types of infant formula are available that are designed for different ages of infants within the first six months of life.

The digestive system changes tremendously during infancy (Bourlieu et al., 2014), which may affect the digestion of infant nutrition. After birth, activities of digestive enzymes, such as pepsin and trypsin, are low, and the pH in the stomach is relatively high compared to adults. In full-term newborns, the pH in the stomach ranges from 3.5 before to 6.5 after drinking mother's milk, and it takes more than 3h before the preprandial pH values are reached again (Gan et al., 2018; Mason, 1962), whereas in adults, the pH in the stomach ranges from 1.5 before to 6.5 after drinking casein or whey protein solutions, and it takes only 1h before the pH is below 3.5 again (Calbet & Holst, 2004; Gan et al., 2018). During the first months of life, the pH in the stomach slowly decreases from newborn towards more adult values and digestive enzyme activities increase. Although some digestive parameters have already reached adult values directly after birth, such as gastric lipase activity (Bourlieu et al., 2014), other parameters take years before they reach adult values, such as the gastric acid profile (Bowles et al., 2010). Understanding the effect of age-related digestion conditions on the protein digestion of infant nutrition may aid in optimizing infant formula to promote infant growth and health.

Nowadays, two widely used static *in vitro* digestion models exist that mimic adult and infant conditions: the consensus INFOGEST adult digestion model (Brodkorb et al., 2019), and the infant model that represents one-month-old infants (Ménard et al., 2018). By use of these digestion models, caseins were shown to be digested faster and whey proteins more extensively during gastric digestion in the adult compared to the infant model (Ménard et al., 2018). After *in vitro* intestinal digestion of milk proteins, smaller peptides and a lower variety of β-lactoglobulin (β-Lg) and β-casein peptides were detected in adults compared to infants (Torcello-Gómez et al., 2020). The milder digestion conditions in infants thus lead to differences in both gastric and

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intestinal digestion compared to adults. However, obtaining information on how milk protein digestion changes with infant age is difficult since static *in vitro* models for other infant ages are either lacking, do not indicate what infant age they are representing, or are not in line with the current consensus adult and one-month infant digestion models (Shani-Levi et al., 2017).

To set up *in vitro* digestion models representing different ages, *in vivo* data are needed on the digestion conditions of infants at different ages, which has been reviewed by Bourlieu et al. (2014) and has been described in brief above. Such age-related *in vivo* data are available on gastric conditions but are extremely scarce on intestinal conditions because of the invasiveness of obtaining these data. Since this lack of data makes it currently impossible to include accurate intestinal digestion conditions for different age stages during infancy, we focused only on the gastric digestion conditions. Changes in gastric digestion conditions likely influence both gastric and intestinal digestion kinetics and subsequent uptake of amino acids. In addition, the gastric clot may be affected by infant age since the gastric clot was shown to depend on both pH and pepsin concentration (Yang et al., 2022), which change during infancy. The presence and structure of the gastric clot is important for infants to enable a constant release of amino acids into the bloodstream and to avoid nitrogen loss (Diether & Willing, 2019; Huppertz & Chia, 2021; Lacroix et al., 2006).

In addition to digestion conditions, milk protein digestion is also affected by heat treatment (van Lieshout et al., 2020). While mother's milk is usually drunk raw by children, infant formula undergoes heat treatment before consumption to ensure microbiological safety. In raw bovine milk, a firm clot is formed in the stomach, which consists only of caseins. Upon heating, whey proteins unfold and bind to the outside of the casein micelle, resulting in a looser clot that contains both caseins and whey proteins (Ye et al., 2019). Furthermore, native whey proteins are not susceptible to pepsin digestion, whereas denatured whey proteins are digested by pepsin because of an increased accessibility of cleavage sites after unfolding (Wang et al., 2018). Protein denaturation can therefore alter digestion kinetics and amino acid levels in the bloodstream (Barbé et al., 2013).

This study aimed to investigate the effect of age-dependent changes in gastric digestion conditions on milk protein digestion in infants during the first six months of life. As protein denaturation and aggregation are known to play a major role in milk protein digestion, changes in digestion of both unheated and heated milk proteins during different stages of infancy were studied. We hypothesized that milk proteins are more extensively digested with increasing age due to maturation of the gastric digestion conditions, and that heated milk proteins form a looser gastric clot and are therefore digested faster than unheated milk proteins at all age stages because of the previously shown increase in whey protein digestion after denaturation.

2. Materials and methods

2.1. Materials and chemicals

Raw cow's milk was provided by FrieslandCampina (Wageningen, The Netherlands). Pierce BCA protein assay kit, NuPAGE LDS sample buffer (4x), NuPAGE sample reducing agent (10X), NuPAGE 4 to 12% Bis-Tris protein gel, and NuPAGE MES SDS running buffer (20X) were purchased from ThermoFisher Scientific (Massachusets, USA). BlueRay prestained protein marker was obtained from Jena Bioscience (Jena, Germany). Pepsin (P6887), pepstatin A (P5318) and all other chemicals were purchased from Sigma Aldrich (Missouri, USA).

2.2. Processing of milk

As human milk is usually only drunk raw and commercial infant formulas have undergone heat treatments, which will result in variable levels of whey protein denaturation, raw cow's milk was chosen as starting material to enable studying differences in digestion between native and denatured proteins. To skim the raw cow's milk, it was centrifuged at 6000g at 4°C for 20 min, after which the cream layer was removed. Thereafter, part of the skim milk was heated in a water bath at 80°C. Once the skim milk had reached this temperature, which took about 15 min, heating was continued for 30 min. This heating temperature and duration was chosen to induce denaturation and aggregation, while avoiding the formation of other protein modifications, such as glycation as shown by Wu et al. (2023). After heating, the skim milk was cooled down by placing it in ice water. The skim milk was stored at −20°C until further use.

2.3. In vitro gastric digestion models

2.3.1. One-month infant gastric digestion model

The gastric phase of the infant static digestion model of Ménard et al. (2018) was used to mimic the gastric digestion of one-month-old infants without the addition of gastric lipase since skim milk was used for the digestion experiments. This one-month (1M) model uses a meal:simulated gastric fluid (SGF) ratio of 63:37, a pH value of 5.3, and a pepsin activity of 268 U/ml. This meal:SGF ratio and this pH value are based on the gastric conditions at the emptying half-time of infants after drinking infant formula (78 min; Cavell, 1981) since the gastric emptying half-time is usually considered more relevant than the final gastric digestion timepoint. The duration of the gastric digestion was set to 60 min, which is close to the gastric emptying half-time. The pepsin activity of 268 U/ml in the final mixture of meal and SGF was calculated using the mean body weight of one-month-old infants and the average pepsin activity (63 U/ml gastric content/kg body weight) measured in infant gastric aspirates (Armand et al., 1996). SGF consisted of 94 mM sodium chloride and 13 mM potassium chloride, and was based on the gastric fluid composition of full-term infants (Hyde, 1968).

2.3.2. Three-month and six-month infant gastric digestion models

The 1M digestion model was used as a basis, and its parameters were adapted to set up gastric digestion models representing three-month-old and six-month-old infants (Table 1). The parameters of the two models were determined based on the same gastric emptying half-time as used in the 1M model (78 min) because the half-time does not change during the first year of life (Billeaud et al., 1990). In addition, it is still unknown how the gastric acid composition and production develop during the first two years of life. Therefore, the SGF composition, meal:SGF ratio, and the duration of the gastric digestion were used according to the 1M model in all three digestion models. For the three-month (3M) and six-month (6M) models, pH was set to 4.7 and 4.4 and pepsin activity to 385 U/ml and 480 U/ml in the final mixture of meal and SGF, respectively. The chosen pH values are the averages that were found in an *in vivo* study in which the pH value was measured in the stomach of 2–3 month-olds and 4–6 month-olds one hour after they were fed dried skim milk (Miller, 1942). The used pepsin activities of 385 and 480 U/ml were calculated in the same manner as for the 1M model (Ménard et al., 2018), in which the mean body weight and average pepsin activity (63 U/ml gastric content/kg body weight) measured in infant gastric aspirates (Armand et al., 1996) were used. With a mean body weight of 6.11 kg for three-month-old infants and 7.62 kg for six-month-old infants (WHO, 2006), this results in a pepsin activity of 385 U/ml and 480 U/ml

Table 1
Parameters of the *in vitro* infant gastric digestion models representing one-month (1M), three-month (3M) and six-month (6M) -old infant digestion.

Digestion model	1M	3M	6M
Ratio meal: SGF	63:37	63:37	63:37
pH	5.3	4.7	4.4
Pepsin activity (U/ml)	268	385	480
Duration (min)	60	60	60

in the final mixture of meal and SGF, respectively. Gastric lipase was not added in this study since skim milk was digested but a lipase activity of 21 U/ml would be suitable for three- and six-month-old infants. This activity is identical to the lipase activity in the INFOGEST adult model (Brodkorb et al., 2019) because gastric lipase has already reached adult activity levels upon birth (Bourlieu et al., 2014).

2.4. In vitro gastric digestion

In vitro gastric digestions were performed using the three digestion models representing one-month, three-month and six-month-old infants as described above. Unheated skim milk (USM), heated skim milk (HSM) and SGF were preheated at 37°C for 10 min. Thereafter, SGF was added to the milk, pH was adjusted using 1M HCl, and pepsin was added. These mixtures were incubated at 37°C with continuous shaking of 20 rpm. Digestions were stopped after 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, G60) by the addition of 10 μl of 7.2 μM pepstatin per mL digest. After digestion, samples for soluble protein content, protein composition, and free amino groups measurements were centrifuged at 4500g at 4°C for 30 min. The supernatant (soluble digest) was poured into a new tube, and the supernatant and the remaining pellet (gastric clot) were stored separately at −20°C until further use. Different digestion samples were prepared for confocal laser scanning microscopy (CLSM) and dry matter content, which were only digested for 60 min. These samples were not centrifuged after digestion to retain the gastric clot structure but were placed on ice and were used on the same day for further preparation for analysis. Digestions were performed in triplicate.

2.5. Soluble protein

Protein concentration of the soluble digests was measured with the BCA assay following the manufacturer's protocol. Percentage soluble protein was calculated by the following formula:

$$Soluble\ protein\ (\%) = \frac{Measured\ soluble\ protein\ concentration}{Theoretical\ protein\ concentration} \bullet 100\%$$

in which measured soluble protein concentration is the protein concentration measured with the BCA assay and theoretical protein concentration is the protein concentration in milk before digestion.

2.6. Dry matter content of clots

Dry matter content of the clots at G60 was determined by measuring the wet and dry weight of the clots. Approximately 1 g of clot was weighed and was dried at 100°C for 24 h. After drying, the clot was weighed to determine the dry weight. Dry matter content of the clot was calculated by use of the following formula:

$$Dry\ matter(\%) = \frac{Dry\ weight}{Wet\ weight} \bullet 100\%$$

in which dry weight is the weight of the clot after drying and wet weight is the weight of the clot before drying.

2.7. Microstructure of clots

The microstructure of the clots at G60 was visualized by use of confocal laser scanning microscopy (CLSM). After digestion, clots were washed twice by pouring off the liquid and adding fresh SGF at the same pH as the corresponding digestion model. Proteins in the clots were stained overnight by the addition of Rhodamine B (0.2% w/v). The next day, the residual dye was removed and proteins in the clots were visualized by use of an LSM510 microscope (Zeiss) with an excitation wavelength of 542 nm, and emitted light was collected between 540–750 nm. Images of the clots were taken with a magnification factor of 20 using Zen 2009 software.

2.8. SDS-PAGE

SDS-PAGE was performed to monitor the disappearance of intact protein in the clots and soluble digests during gastric digestion. Clots were freeze-dried. Approximately 0.22 mg freeze-dried clots were mixed with 25 μl 4x LDS sample buffer, 10 μl 10x reducing agent, and 65 μl Milli-Q water. Soluble digests were mixed with 4x LDS sample buffer, 10x reducing agent, and Milli-Q water in a 1/5/2/12 (v/v/v/v) ratio. Subsequently, both soluble digest and clot samples were heated at 70°C for 10 min. After heating, a volume corresponding to 5 μg protein of clots and soluble digests, as measured with the BCA assay, and 3 μl marker were loaded on a 4–12% Bis-Tris polyacrylamide precast gel. Gels were run at 120V for 75 min with MES running buffer, stained with Coomassie brilliant blue solution, and destained with 10% (v/v) ethanol and 7.5% (v/v) acetic acid solution. Bands on the gels were visualized by use of a GS-900 Calibrated Densitometer (Bio-Rad) with Image Lab software.

2.9. Proteomics

Bands of interest on the SDS-PAGE gels were analyzed further on protein composition by use of LC-MS/MS as described previously (Xiong et al., 2021) with some modifications. After destaining, gels were washed three times with Milli-Q water. Then, gels were reduced by 15 mM dithiothreitol for 1h and alkylated by 20 mM acrylamide for 30 min. Gels were washed five times with Milli-Q water, and bands were excised from the gel and cut into small pieces. Gel pieces were digested with 50 μl trypsin (5 ng/μl) overnight at room temperature. Then, 10% trifluoro acetic acid was added to lower the pH to 2–4. Thereafter, samples were cleaned up with Solid Phase Extraction (SPE) C8 stage tip columns, which were made in-house. Samples were loaded onto the C8 stage tip columns, and peptides were eluted into low-binding Eppendorf tubes. To extract more peptides from the remaining gel pieces, 100 μl of 50% acetonitrile/ 50% 1 ml/L formic acid was added to the remaining gel pieces, mixed and the liquid was added to the C8 filter. Peptides were eluted into the same low-binding Eppendorf tube and were concentrated to a volume of<15 μl with the Eppendorf concentrator at 45°C. The volume of the sample was adjusted to 50 μl with 1 ml/L formic acid. Peptides were analyzed on a Thermo nLC 1000 system (Thermo, Waltham, MA, USA) coupled to an Orbitrap Exploris 480 (Thermo, Waltham, MA, USA). LC-MS/MS parameters were used, and data were analyzed as described previously (Xiong et al., 2021) with a few modifications. Obtained LC-MS/MS data were analyzed with MaxQuant (Cox & Mann, 2008) (v2.0.3.0) with the trypsin semi-specific digestion mode and further default settings in the Andromeda search engine. Modifications that were included in protein quantifications were M−oxidation, protein *N*-terminal acetylation, and N- and Q-deamidation. Maximum number of modifications in one peptide was set to 3. Peptides were identified with the UP000009136 bovine database and a contaminant database that includes common contaminants such as trypsin and human keratin. Intensity values were used to calculate percentages of milk proteins in the SDS-PAGE bands.

2.10. Protein hydrolysis in soluble digests

The o-phthaldialdehyde (OPA) assay was performed to measure free amino groups in the soluble digests, as described previously (Mulet-Cabero et al., 2017). In brief, soluble digests were diluted in Milli-Q water till a protein concentration between 1 and 5 mg/ml, and an L-leucine standard curve ranging from 5 to 50 μM was prepared. Then, 200 μl of freshly prepared OPA reagent was added to 10 μl diluted sample, or L-leucine standard in a transparent 96-well polystyrene plate. After incubation in the dark for 15 min, absorbance was measured at 340 nm with a Spectramax M2 microplate reader (Molecular Devices). Finally, protein hydrolysis was calculated using the following formula:

$$\text{Protein hydrolysis} = \frac{\textit{Free } NH_2 \textit{ groups in soluble digest } (mM)}{\text{Protein concentration in soluble digest (mg/ml)}}$$

in which Free NH_2 groups in soluble digest is the concentration of free amino groups in the soluble digest as determined by the OPA assay, and protein concentration in soluble digest is the protein concentration in the soluble digest as determined by the BCA assay.

2.11. Statistical analysis

Statistical analysis was performed in GraphPad prism v8.0.2 (GraphPad Software, San Diego, California USA). As not enough data were obtained to perform a normality test, normality of the data was assumed. Comparison between 1M and 3M and between 1M and 6M models was done using one-way ANOVA and Dunnett's multiple comparisons test. Comparison between different heat treatments was done using unpaired two-tailed *t*-test. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Clot formation and structure during gastric digestion

Formation and proteolysis of gastric clots from USM and HSM during digestion with 1M, 3M and 6M *in vitro* digestion models were monitored. Changes in percentage soluble protein during gastric digestion compared to undigested skim milk were measured with the BCA assay (Fig. 1), which detected both intact proteins and large peptides. At G0, digests from all age models had a soluble protein percentage lower than 100%, and digests from the 3M and 6M models had a lower soluble protein percentage than digests from the 1M model. During the first 5 min of gastric digestion, soluble protein concentration decreased in USM in all models and in HSM in the 1M model, followed by a gradual increase in soluble protein concentration till 60 min. Whereas the percentage soluble protein only slightly increased in the 1M model between 5 and 60 min of digestion in both USM and HSM, it increased to a greater extent in the 3M and 6M models. At the end of gastric digestion, USM and HSM digests in the 3M and 6M models contained significantly more soluble protein than in the 1M model, and USM digests contained significantly more soluble protein compared to HSM digests in the same digestion model (Fig. 1C).

More information about the gastric clots was obtained by studying their structure and dry matter content at G60 (Fig. S1). Clots from USM in the 1M model seemed firmer than all other clots by visual observation and had the highest dry matter content (19.6%). Compared to the clot from USM in the 1M model, lower dry matter contents were found in the 3M and 6M models (16.2% and 17.9% respectively), although this difference was only significant compared to the 3M model. Clots from HSM had similar dry matter contents in all digestion models, which ranged from 16.8 to 17.2%. Only in the 1M model, clots from USM had a significantly higher dry matter content than those from HSM.

The microstructure of the gastric clots at G60 was analyzed with CLSM (Fig. 2). Microscopy images revealed that clots from USM consisted of large aggregated structures in the 1M and 3M models. The clot structure seemed denser with smaller pores in the 1M model than in the 3M model, which is in line with the higher dry matter content of the USM clots in the 1M model. In the 6M model, small aggregates were observed in USM with a size of around 50–150 μm. In HSM, clots in the

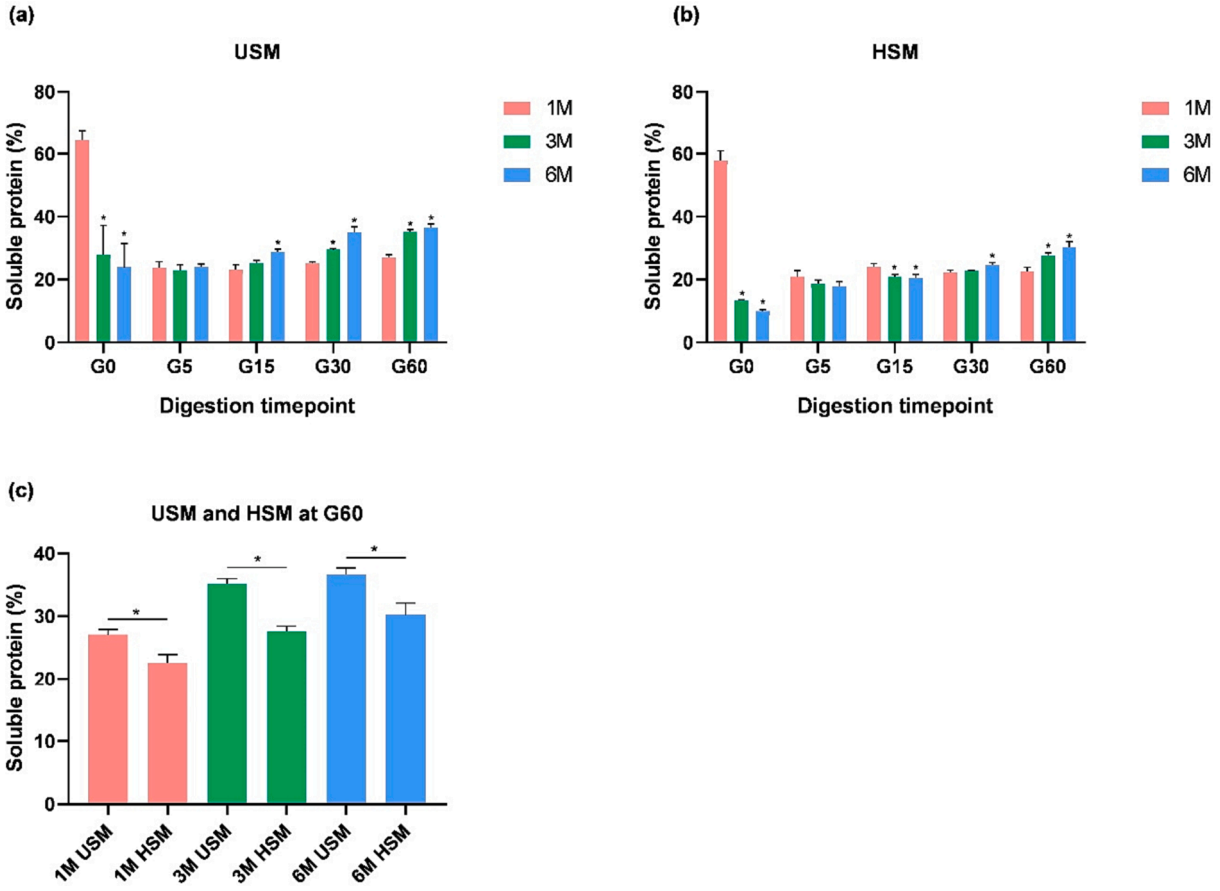

Fig. 1. Percentage soluble protein after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, G60) of (a) unheated skim milk (USM) and (b) heated skim milk (HSM), and (c) comparison of percentage soluble protein at G60 between USM and HSM. Gastric digestion was conducted by use of *in vitro* infant gastric digestion models representing one-month (1M), three-month (3M) and six-month (6M) -old infants. Differences were considered statistically significant at $p < 0.05$.

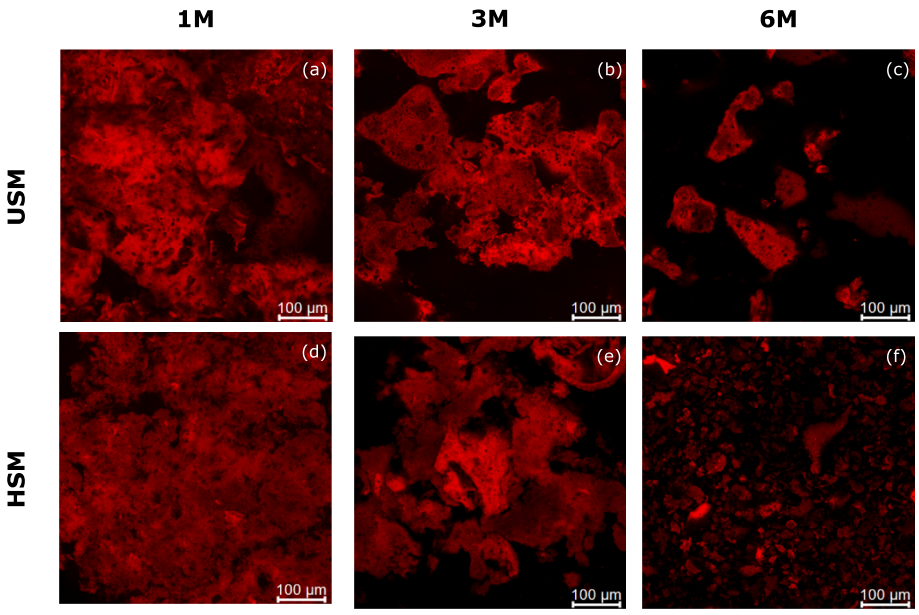

Fig. 2. Confocal Laser Scanning Microscopy (CLSM) images of gastric clots from unheated (USM) and heated skim milk (HSM) after 60 min of gastric *in vitro* infant digestion with models representing one-month (1M), three-month (3M) and six-month (6M) -old infant digestion. Proteins were stained with 0.2% Rhodamine B. Clots from USM after 60 min gastric digestion with (a) 1M (b) 3M and (c) 6M infant digestion models, and clots from HSM after digestion with (d) 1M (e) 3M and (f) 6M infant digestion models.

1M and 3M models also had large aggregated microstructures, whereas clots in the 6M model consisted of many small aggregates with sizes ranging from 20 to 100 µm. Differences between the clots from USM and HSM were found in the 1M and 6M models. In the 1M model, clots from USM showed a higher protein density than clots from HSM, and in the 6M model, clots from USM consisted of larger aggregates than clots from HSM. No clear differences could be observed between the clots from USM and HSM in the 3M model.

3.2. Protein composition of gastric clots

The protein composition of the gastric clots was determined by use of SDS-PAGE, and ten bands were analyzed for protein identification with LC-MS/MS (Fig. 3). At G0, casein bands in the 3M and 6M models were more intense than in the 1M model, showing that relatively more casein was incorporated in the clots in the 3M and 6M models. β- and κ-casein were digested the quickest, as nearly no intact protein was detected with LC-MS/MS after 5 min of digestion (band 3), whereas α_{s2}-casein (band 1) was the most persistent during digestion, as some intact casein was still present after 60 min of digestion in all digestion models. Intact caseins disappeared faster from the clots in the 3M and 6M models than in the 1M model on the SDS-PAGE gel. Caseins were digested into large peptides with varying sizes (bands 2–4, 7, 9 and 10), which showed an increased intensity in the 3M and 6M models compared to the 1M model. The bands with a higher molecular weight (bands 2–4) consisted of a mixture of α_{s2}-, α_{s1}- and β-caseins, whereas bands with a smaller molecular weight (bands 7, 9 and 10) consisted of a mixture of α_{s2}-, α_{s1}- and κ-casein and β-Lg peptides. The total composition and intensities of the proteins in the bands analyzed with LC-MS/MS are shown in Table S1.

In addition to caseins, also intact whey proteins were detected in the clots in all digestion models, which remained largely intact during gastric digestion. Relative band intensities of β-Lg (band 5) and α-lactalbumin (α-La; band 8) at G60 were either similar or increased compared to at G5, showing that they were digested slower than caseins. Only in the 6M model, band intensities of β-Lg and α-La slightly decreased during digestion. The digestion of β-Lg resulted in peptides ranging from $<$ 8 to 17 kDa (bands 6 – 10), whereas no α-La peptides were detected in the clots. The effect of heating could also be observed in the protein composition of the clots. Clots from HSM contained relatively more whey proteins (bands 5 and 8) than clots from USM already at G0, and this difference persisted till the end of gastric digestion. Heating also resulted in a slower disappearance of intact α_{s2}-casein but this effect was more pronounced in the 1M model than in the 3M and 6M models. Moreover, clots from HSM showed higher band intensities of small peptides (bands 9 and 10) in all digestion models.

3.3. Soluble protein composition during gastric digestion

Intact caseins disappeared from all soluble digests within the first 5 min of digestion due to clot formation (Fig. 4). With increasing age, whey proteins such as β-Lg, α-La, lactoferrin (Lf), bovine serum albumin (BSA) and immunoglobulins, were digested faster in both USM and HSM, although intact whey proteins were still present at the end of gastric digestion in all models. Furthermore, an increased intensity of small peptides was observed with increasing age. Heating resulted in a decreased amount of whey protein in the soluble digests at G0 in the 3M and 6M models and at G5 in the 1M model, and in a larger decrease in band intensities of the whey proteins during digestion.

Five bands on the SDS-PAGE gel were analyzed with LC-MS/MS to determine their protein composition. At G0, two bands (bands 11 and 12) were selected that did not match the molecular mass of intact milk proteins. These bands consisted of α_{s1}-, α_{s2}- and β-casein as well as β-Lg. At G5, a faint band with a size of 25 kDa (band 13) became visible in USM and remained present till the end of gastric digestion, which contained Ig-like domain-containing protein and β-Lg. Peptides with a lower molecular weight (bands 14 and 15) were present from G5 onwards and consisted of α_{s1}-, α_{s2}-, β-casein and β-Lg. Peptides smaller than 8 kDa, originating from α_{s1}- and α_{s2}-casein and β-Lg, were thus detected in both the clot and soluble digest, whereas small peptides from β-casein were mainly detected in the soluble digest, and small peptides from κ-casein were mainly detected in the clot.

3.4. Hydrolysis of soluble protein during gastric digestion

Free amino groups were determined in the soluble digests and were corrected for soluble protein concentration as a measure of protein hydrolysis (Fig. 5). Protein hydrolysis increased quickly during the first 15 min and more slowly during the following 45 min of gastric digestion. Compared to the 1M model, protein hydrolysis was increased in the 3M and 6M models for both USM and HSM. Although significant differences between the different digestion models were observed in USM digests at G15 and in HSM digests at G5, G15 and G30, only between the 1M and 3M models in HSM digests the protein hydrolysis significantly differed at the end of gastric digestion. However, no differences in protein

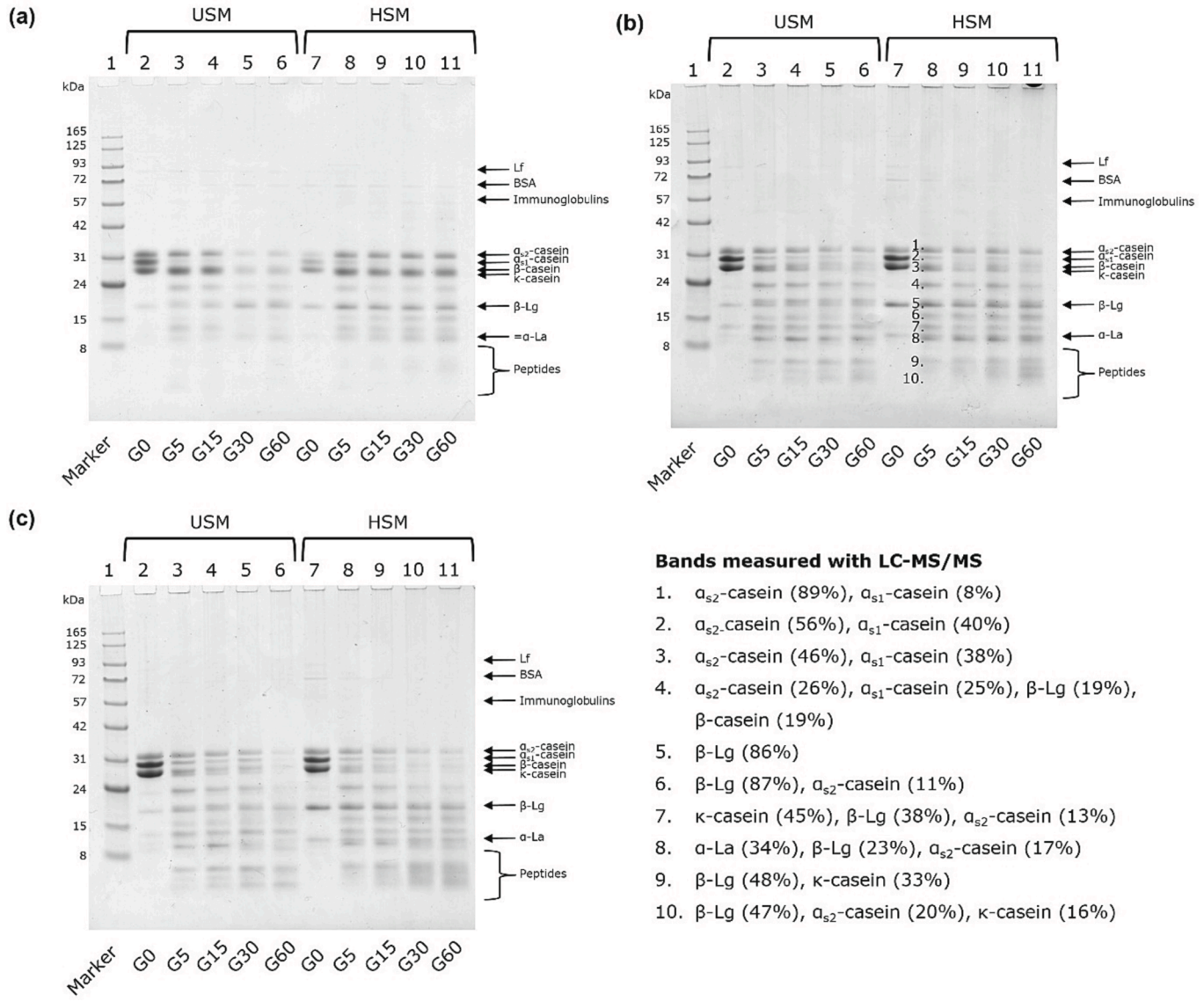

Fig. 3. SDS-PAGE gels of gastric clots from unheated (USM) and heated skim milk (HSM) after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, G60) with digestion models representing (a) one-month (1M), (b) three-month (3M), and (c) six-month (6M) -old infant digestion. The numbered bands in the gels were analyzed on protein composition with LC-MS/MS.

hydrolysis at G60 were found between soluble digests from USM and HSM that were digested with the same digestion model (Fig. 5C).

4. Discussion

This study aimed to determine age-dependent changes in gastric clot formation and digestion of milk proteins in infants by use of *in vitro* gastric digestion models representing one-month, three-month and six-month-old infants. Results showed that gastric clot formation and clot structure differed between the models and that milk proteins were digested faster with increasing age due to age-dependent maturation of gastric digestion conditions.

4.1. Age-dependent effect on gastric clot formation and breakdown

Age-dependent differences were observed for the formation and breakdown of the clots. Less protein was incorporated in the clots from USM and HSM in the 1M model at G0 compared to the 3M and 6M models (Fig. 1). According to the SDS-PAGE results of the gastric clots (Fig. 3) and the soluble digests (Fig. 4), the clots in the 1M model also had a lower casein content and the soluble digests had a higher casein content than the 3M and 6M models. At G0, gastric clot formation was only acid-induced as no pepsin was present at this time point. More acid-induced coagulation took place in the 3M and 6M models because their pH values (4.7 and 4.4) were closer to the isoelectric point (pI) of caseins (4.6), whereas the pH value of the 1M model (5.3) deviated more from the pI of caseins. Clots from HSM contained more protein than those from USM at G0, which can probably be attributed to a higher content of whey protein in these clots as shown by SDS-PAGE (Fig. 3). During heat treatment, whey proteins denature and bind to the outside of casein micelles, resulting in gastric clots that consist of both caseins and whey proteins (Ye et al., 2016). However, clots from USM also contained some whey protein at G0, demonstrating that also native whey proteins were present in gastric clots. Whereas a very low level of glycation may have occurred during wet heating at 80°C for 30 min (Wu et al., 2023), such low glycation levels have been shown not to influence gastric milk protein digestion (Zenker et al., 2020). Therefore, changed milk protein digestion after heat treatment was considered to be caused solely by protein denaturation and aggregation.

Further clot formation and breakdown of the clot occurred between 5

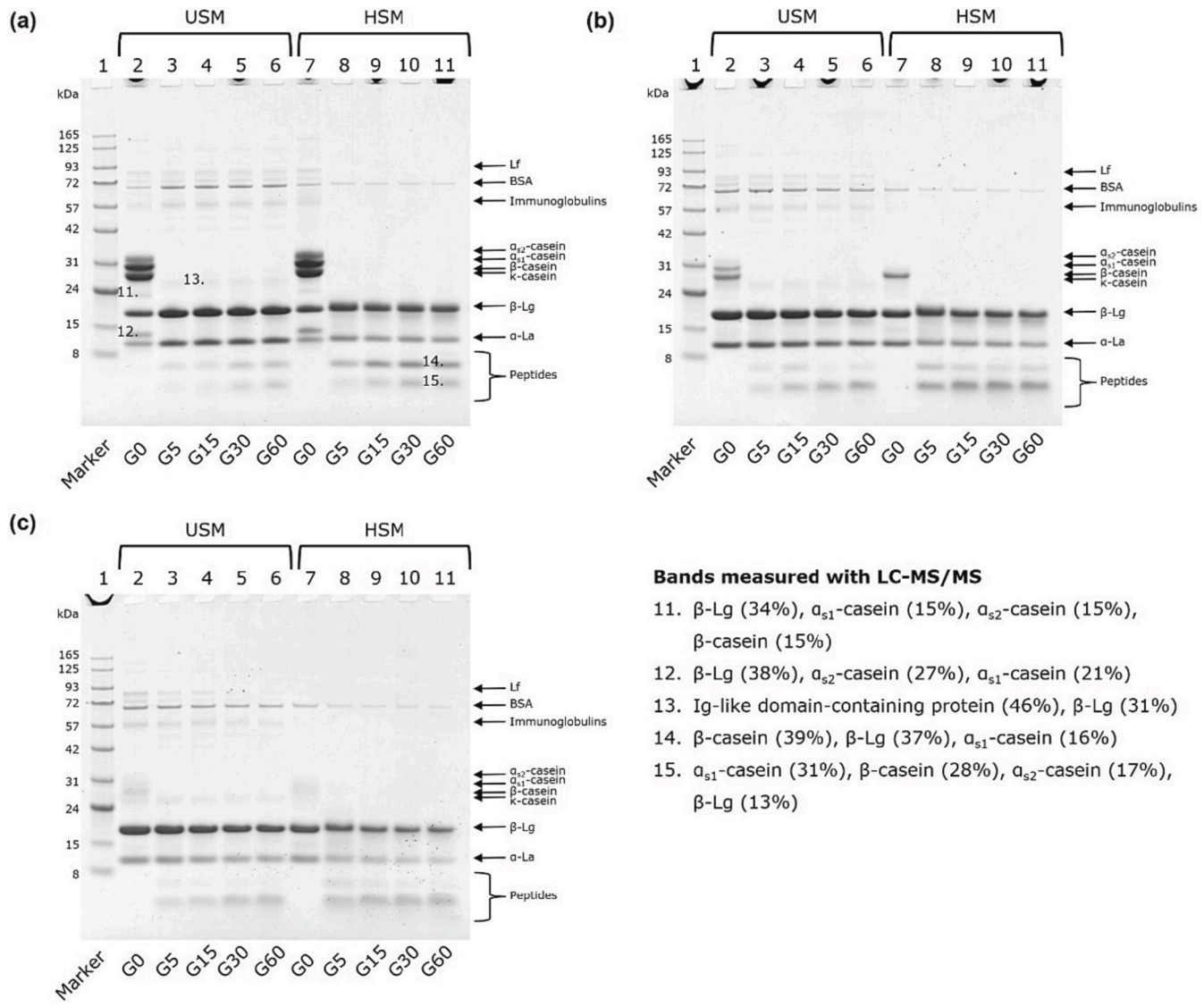

Fig. 4. SDS-PAGE gels of soluble digests from unheated (USM) and heated skim milk (HSM) after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, G60) with digestion models representing (a) one-month (1M), (b) three-month (3M), and (c) six-month (6M) -old infant digestion. The numbered bands in the gels were analyzed on protein composition with LC-MS/MS.

and 60 min due to pepsin activity. The soluble protein content was similar for all digestion models at G5 (Fig. 1). Thereafter, the amount remained similar till the end of digestion in the 1M model, whereas it increased for the 3M and 6M models. This indicates that gastric clots were broken down faster, but gradually, in the 3M and 6M models, which may be caused by the faster digestion of caseins as visualized with SDS-PAGE (Fig. 3). A gradual digestion of the clot was shown to be important in infants to provide a constant release of amino acids after intestinal digestion without leading to nitrogen loss (Huppertz & Chia, 2021; Lacroix et al., 2006). The faster clot breakdown in the 3M and 6M models was probably caused by the higher pepsin concentrations and lower pH values than the 1M model, which both contribute to a higher pepsin activity, leading to a quicker digestion of the caseins into peptides and subsequent breakdown of the clot. While the pepsin concentration of the 6M model (480 U/ml) was not even twice as high as the one of the 1M model (268 U/ml), the pepsin activity differed more. At the pH of the 6M model (4.4), pepsin is at 65% of its maximal activity, whereas at the pH of the 1M model (5.3), this is only 10% (Piper & Fenton, 1965). Therefore, the pepsin activity was about twelve times higher in the 6M model compared to the 1M model, resulting in a faster gastric protein digestion with increasing age.

The difference in clot structure and hydration might also have contributed to the quicker breakdown of the clot with increasing age. The clot from USM in the 1M model had a higher dry matter content than clots in the 3M and 6M models, meaning a lower hydration (Fig. S1). This was in agreement with the higher protein density that was observed for the USM clot in the 1M model with CLSM (Fig. 2). This lower hydration can lower the accessibility for pepsin, which may result in a slower breakdown of the clot. Clots from the 6M model consisted of smaller aggregates as determined by CLSM (Fig. 2), which thus have an increased relative surface area compared to larger aggregates, increasing the accessibility for pepsin and enhancing the breakdown of the clot. Microstructures of pepsin-induced milk clots were previously shown to depend on pH value, as clots formed at the same pepsin activity had larger pore sizes at a pH of 5.3 than at higher pH values (Yang et al., 2022). The faster digestion of gastric clots with a higher hydration and a more fragmented microstructure was in line with Ye et al. (2016), although in their study it was caused by heat treatment of milk prior to digestion instead of by different pH values and pepsin activities during digestion.

The faster digestion of milk proteins with increasing age was also shown by the difference in protein and peptide composition in the clot

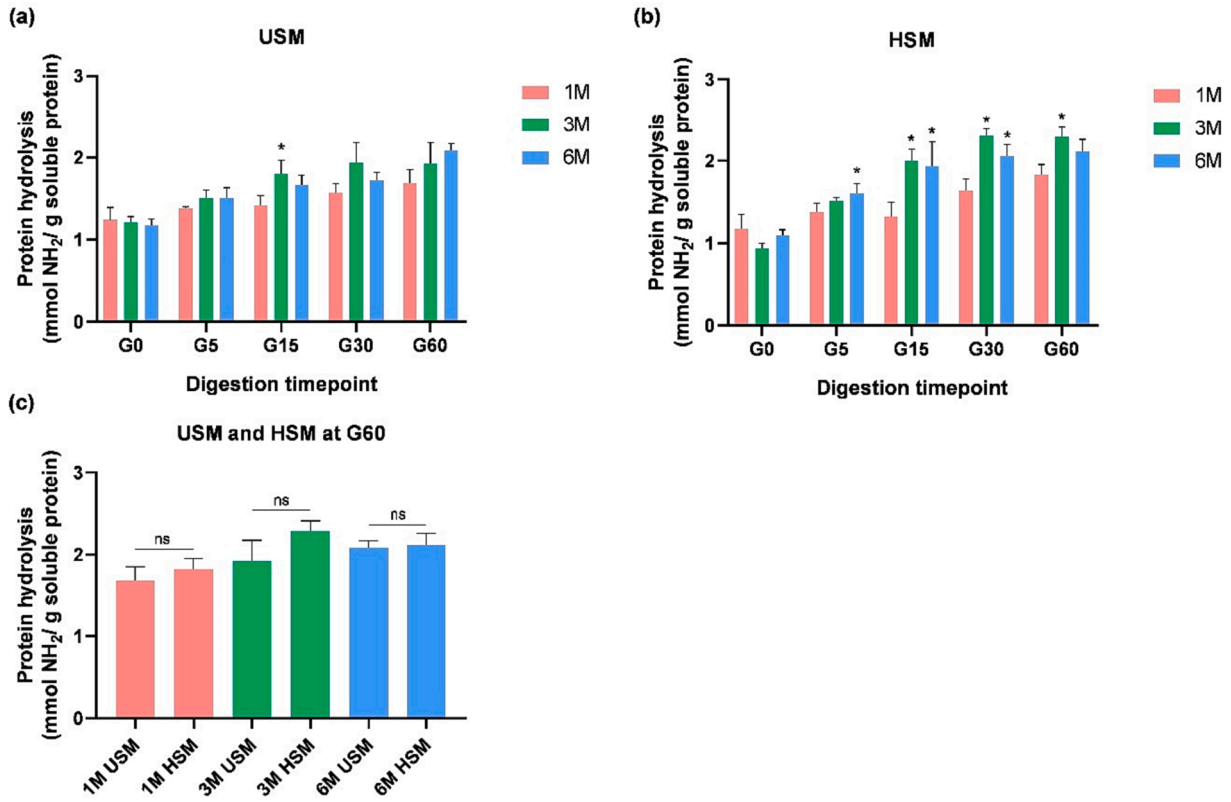

Fig. 5. Protein hydrolysis measured as free amino groups (NH_2) per gram soluble protein after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, G60) of (a) unheated skim milk (USM), (b) heated skim milk (HSM), and (c) comparison of protein hydrolysis at G60 between USM and HSM. Gastric digestion was conducted by use of *in vitro* infant gastric digestion models representing one-month (1M), three-month (3M), and six-month (6M) -old infants. Differences were considered statistically significant at $p < 0.05$ with ns meaning non-significant.

between the three age models (Fig. 3). Intact caseins disappeared faster but still gradually from the clots with increasing age, resulting in more peptides with different sizes in the 3M and 6M models compared to the 1M model. In addition, whey proteins were digested faster in the clots from the 6M model than in those from the other models, although still to a small extent. In contrast to caseins, whey proteins are largely resistant to pepsin cleavage. Therefore, caseins were affected to a greater extent by the increased pepsin activity with increasing age. This is in agreement with Ménard et al. (2018), who reported a gradual decrease of intact casein during infant gastric digestion and a quick decrease during adult gastric digestion. Peptides that were detected in the clots originated from α_{s1}-, α_{s2}-, β- and κ-casein and β-Lg. These peptides were probably hydrophobic as they did not solubilize. In a previous study on the gastric clot of bovine skim milk, also peptides with different sizes were detected with SDS-PAGE including *para*-κ-casein (Ye et al., 2016). However, by use of SDS-PAGE not all peptides in the clot could be identified in that study. In one study, the protein composition of gastric clots from goat skim milk was analyzed with SDS-PAGE in combination with LC-MS/MS (Ren et al., 2023). They also found that large peptides in the gastric clots were mainly originating from α_{s1}-, α_{s2}- and β-casein, whereas the smaller peptides were from α_{s1}-, α_{s2}- and κ-casein as well as whey proteins.

Relatively larger differences were observed between the digestion models for HSM than USM with regard to casein digestion and peptide formation (Fig. 3). The higher increase in peptide intensity in HSM might be caused by the presence of denatured whey protein. Denatured whey protein is more easily digested by pepsin than native whey protein because of unfolding, leading to an easier access to the cleavage sites (Tunick et al., 2016). The increased whey protein digestion after heating might cause bigger differences in peptide formation with age-dependent increasing pepsin activity. Heating also led to a slower casein digestion but only in the 1M model. The clot of USM visibly had a firmer clot structure, a higher dry matter content (Fig. S1), and a higher protein density (Fig. 2) than the clot of HSM in the 1M model, whereas no differences were observed between USM and HSM in terms of dry matter content and protein density in the other models. The higher resistance of casein to infant gastric digestion in HSM than USM is in line with a previous study, which was suggested to be due to the formation of casein-whey aggregates blocking the casein cleavage sites (Sánchez-Rivera et al., 2015). In another study, also firmer gastric clots were reported for unheated milk than for heated milk (Ye et al., 2016). However, they found a faster instead of a slower casein digestion after heating due to the looser clot structure, which was more susceptible to pepsin cleavage. This difference may be explained by the use of a dynamic digestion model that included mechanical disruption of the gastric clot, which is not included in static digestion models as used in our study. Overall, gastric digestion of both USM and HSM increased with age but the difference was larger for HSM than USM, which was most likely caused by differences in pepsin activity and gastric clot structure.

4.2. *Age-dependent effect on hydrolysis of soluble protein*

Differences in milk protein digestion between the different age models were also observed in the soluble digests. With increasing age, clots were digested faster, leading to a higher soluble protein content (Fig. 1). In addition, whey proteins were more easily digested and smaller peptides were formed (Fig. 4), indicating a more extensive protein digestion with increasing age. The soluble protein composition of USM digested with the 1M model was in agreement with Ménard et al. (2018), in which at least 80% of the whey proteins were still intact after 60 min of gastric digestion. The protein compositions of the 3M and 6M digests were in between the protein compositions of digests from the 1M

model of Ménard et al. (2018) and the adult INFOGEST model (Ménard et al., 2018), showing that the gastric milk protein digestion increases during infancy but does not reach adult conditions yet. Whereas nearly all whey proteins had been digested after 60 min in the adult model, a large part of the whey proteins was still intact after digestion with the 3M and 6M models. Peptides from α_{s1}-, α_{s2}- and β-casein were detected in the soluble digests. As no intact caseins were present in the soluble digests after 5 min of digestion, these peptides most likely moved from the gastric clot to the soluble digests, showing a gradual release of casein peptides from the clot during gastric digestion. Peptides from β-Lg were also detected in the soluble digests and could have originated from both β-Lg in the clot as from soluble β-Lg.

An increased digestibility with increasing age was also seen in protein hydrolysis for HSM but not for USM (Fig. 5), while an increased percentage soluble protein with increasing age was observed for both USM and HSM (Fig. 1). The higher percentage soluble protein with increasing age was probably due to the difference in pepsin activity between the age models, resulting in more protein solubilizing from the gastric clot from both USM and HSM to the soluble digest. An increased protein hydrolysis with increasing age was, however, only observed in HSM, which means relatively more free NH_2 groups per gram soluble protein. This is probably caused by the larger increase in whey protein digestion with age in HSM than in USM, due to the easier digestion of denatured compared to native whey protein (Wang et al., 2018), resulting in relatively more small peptides with more free NH_2 groups in HSM. This is in agreement with the faster breakdown of denatured whey protein in the digests and the higher increase in intensity of peptides with age in HSM compared to USM (Fig. 4). Although the HSM digests in the 3M model had a higher protein hydrolysis at some timepoints than in the 6M model (Fig. 5), they did not significantly differ, indicating that the digestion of milk proteins was more affected by the changes in gastric digestion conditions in the first three months of life.

4.3. Relevance and limitations of gastric digestion models

In this study, the effect of infant age on clot formation and digestion of milk proteins during gastric digestion was investigated by use of gastric digestion models representing one-, three-, and six-month-old infants. Studying gastric clot formation and breakdown provides insights into the complete digestion process, as it is known to influence intestinal protein digestion and subsequent uptake of amino acids (Diether & Willing, 2019; Huppertz & Chia, 2021; Lacroix et al., 2006). The 1M model is a model that is commonly used and is in line with *in vivo* gastric data from preterm infants (Henderson et al., 2001), although a direct validation of the *in vitro* model with *in vivo* data from full-term infants is still lacking. The parameters of this 1M model were changed to mimic the digestion of three- and six-month-old infants, which were based on *in vivo* data. However, it remains unknown how well the 3M and 6M models mimic *in vivo* gastric digestion of milk proteins as to our knowledge no *in vivo* studies have been performed that investigated milk protein digestion at different infant ages. Validating the *in vitro* data with *in vivo* data in future studies is needed to determine the accuracy of the gastric digestion models and should be done with either human milk or infant formula as a meal. Obtained *in vivo* data could also be used to improve the gastric digestion models, for instance with age-dependent gastric fluid composition and meal:gastric fluid ratio. Moreover, *in vivo* kinetics of gastric clot formation and breakdown could be more closely mimicked by developing dynamic infant gastric digestion models representing different ages as dynamic gastric acid and enzyme secretions and gastric peristalsis, which change with infant age, may influence the formation, structure and breakdown of the gastric clot. *In vivo* data on digestion kinetics of milk proteins at different infant ages are, however, even more scarcely available, which limits the development of such dynamic *in vitro* digestion models. In addition, no intestinal phase was included in the 3M and 6M models because too little data were available on intestinal digestion conditions at these ages, as explained in the introduction. When more intestinal data would become available, an intestinal phase could be added to the gastric digestion models, and could be used to determine how the found age-related differences in gastric milk protein digestion influence the overall gastro-intestinal digestion at different age stages during infancy.

5. Conclusion

Parameters of an existing static *in vitro* infant gastric digestion model representing one-month-old infants were changed to set up digestion models representing three- and six-month-old infants. Digestion of USM and HSM with these gastric digestion models showed that milk proteins in gastric clots and soluble digests were more intensively digested with increasing age, and this difference with increasing age was larger for HSM than for USM. Both the higher pepsin activity and looser clot structure may have contributed to the faster casein and whey protein digestion with increasing age. The larger difference in protein digestion with increasing age for HSM than for USM may have been caused by a larger difference in the digestion of denatured whey proteins than native whey proteins between the different age models. Together, this demonstrates that milk protein digestion in infants is affected by age-related gastric digestion conditions and that heat treatment influences milk protein digestion differently depending on the gastric pH and pepsin activity. By use of these gastric digestion models, information could be obtained on age-dependent differences in milk protein digestion in infants to optimize infant formulas for specific ages, which better resemble human milk of different lactation stages and its digestion, and better support the infant's development in the first six months of life. However, further investigation on *in vivo* validation of digestion models representing different infant ages is needed.

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CRediT authorship contribution statement

Julie Miltenburg: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Shanna Bastiaan-Net:** Conceptualization, Supervision, Writing – review & editing. **Tamara Hoppenbrouwers:** Conceptualization, Supervision, Writing – review & editing. **Harry Wichers:** Conceptualization, Supervision, Writing – review & editing. **Kasper Hettinga:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137209.

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