Heating affects protein digestion of skimmed goat milk under simulated infant conditions

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ABSTRACT

The objective of this research was to analyse the effects of heating on digestion of skimmed goat milk proteins. Most previous goat milk digestion studies evaluated the digestion only based on the supernatant. In this study, digestion of skimmed goat milk was studied in both supernatant and gastric clot. The results indicated that, compared to mild temperature heated samples (≤75 °C), samples heated at ≥80 °C showed more extensive gastric clot formation with a higher protein digestion rate, but also resulted in a larger amount of undigested whey proteins due to its severe aggregation. For the peptidome, β-casein was the major source of bioactive peptides. The samples heated at 65 °C showed higher bioactive peptide abundances, whereas at temperatures higher than 75 °C, it was reduced due to cleavage into smaller peptides. Overall, this study showed that different heating temperatures induced different whey protein denaturation degrees, which affected their digestion in skimmed goat milk.

1. Introduction

Among the different dairy products, goat milk has recently received more attention because of its high nutritional value and easy digestion (Almaas et al., 2006; López-Aliaga, Díaz-Castro, Alférez, Barrionuevo, & Campos, 2010). While human milk is preferred for feeding the infant, breastfeeding may not be possible and infant formula can then be used to meet the infants’ nutritional needs. Among the different infant formulas available, goat milk-based products have become popular, as it has been reported to be more easily digestible compared to bovine milk-based products, they can also modify the structural properties of goat milk proteins, which may influence their digestion. Therefore, it is important to study the effects of heating on goat milk proteins from both structural and functional perspectives, especially on their digestibility for infants.

The following key proteins are more abundant in goat milk than in cow milk: β-casein (β-CN) (68 % vs 42 %), α52-casein (α52-CN) (18 % vs 13 %), κ-casein (κ-CN) (13 % vs 10 %) and α-lactalbumin (α-LA) (5 % vs 2 %) (Zhao, Zhang, et al., 2020, Farrell et al., 2004), with large variations in protein composition reported between studies. The influence of heating on the protein composition and properties of cow milk have been extensively studied. In comparison, little is known about the effects of heating on goat milk. Based on the protein compositional differences, functional differences would be expected, which may also be related to differences in heat-induced changes between these two types of milk.

Heating at pasteurization conditions (65–95 °C) can break hydrophobic interactions, as well as hydrogen and disulfide bonds between the amino acids in milk proteins, resulting in changes of the protein’s secondary and tertiary structure (Branden & Tooze, 2012; Kyte, 1995). The four main changes of milk proteins upon heating include aggregation of whey proteins, aggregation of whey proteins with κ-CN on the surface of the casein micelle, dissociation of κ-CN prior to complex formation, and dissociation of micelle-bound κ-casein-whey protein complexes (Pellegrino, Resmini, & Luf, 1995; van Lieshout, Lambers, Bragt, & Hettinga, 2019). Of these, the aggregation of whey protein with κ-CN is the main change upon heating (Zhao, Zhang, et al., 2020). In cow milk, whey proteins specifically aggregate with κ-CN, whereas in goat milk, they also aggregate with α52-casein because goat α52-casein has cysteine groups that allow such aggregation (Pesic, Barac, Stanojevic, 2004).
Ristic, Macej, & Vrvic, 2012). Compared to cow milk, the whey proteins more easily interact with casein micelles during heating (Pesic et al., 2012; Zhao, Zhang, et al., 2020). On top of that, the whey proteins are denatured to a greater extent in goat milk than in cow milk. Research has shown that goat whey protein showed nearly no denaturation at 65 °C for 30 min while it is totally denatured after heating at 85 °C for 30 min, making this temperature range (65–85 °C for 30 min) interesting in relation to studying digestion (Zhao, Cheng, et al., 2020).

There are several milk protein parameters that have been shown to affect their digestion, including genotype, post-translation modification, and the protein composition, like the casein:whey ratio (Wang, Ye, Lin, Han, & Singh, 2018). The combination of low pH and pepsin will lead to destabilization of caseins, which induces gastric clot formation. Following heat treatment, whey protein bound to casein and large primary whey protein aggregates are trapped in the casein aggregates, and will thus end up in the gastric clot. The protein disappearing in the supernatant may not only be due to its digestion, but the protein may also have precipitated in a gastric clot. The protein composition of the clot is an important indicator to evaluate the effects of heating on digestion. Most goat milk digestion literature focuses on analysing the supernatant without considering the gastric clots, whereas other literature just focuses on the clots structure (Li, Ye, Pan, Cui, Dave, & Singh, 2022). In the present study, not only the protein profile and the detailed identification of proteins in these clots were explored, but also their digestion rate was determined from both the supernatant and gastric clot perspective.

Protein denaturation and aggregation induced by heating can directly change the native protein profile in milk. After digestion, the protein composition, its hydrolysis, and the resulting peptide release will be changed as well (Ye, Cui, Dalgleish, & Singh, 2017). For cow milk, evidence suggests that heated and denatured β-lactoglobulin (β-LG) can be more digestible due to the unfolding and the resulting increased exposure of proteolytic cleavage sites (Guo, Fox, Flynn, & Kindstedt, 1995; Kitabatake & Kinekawa, 1998; Rahaman, Vasiljevic, & Ramchandran, 2017). In contrast, other studies have reported that severe heating (>100 °C), resulting in protein aggregates with tighter structure, reduced cleavage by enzymes, causing a lower digestibility (Carbonaro, Cappelloni, Sabbadini, & Carnovale, 1997; Wada & Lonnard, 2014). For intestinal digestion, the effects of heating on digestibility seem insignificant or even absent (van Lieshout et al., 2019). There is, however, no study reporting the influence of different levels of heating within the pasteurization range on goat milk protein digestion under infant conditions. An in vitro model for infants aged 28 days was used in this study, which is characterised by a higher pH value in the gastric phase and lower enzyme activities compared to an adult model (Menard et al., 2018). The aim of this study is to explore the effects of heating on the digestibility of goat milk proteins in an in vitro infant digestion model. These results may lead to a better understanding of the specific consequences of heating on infant digestion of goat milk protein. This may also support optimization of industrial processing for goat milk infant products with optimal functionality.

2. Materials and methods

2.1. Materials and chemicals

Raw goat milk was provided by Ausnutria B.V. (Zwolle, Netherlands). NuPAGE® LDS sample buffer (4x concentrated), NuPAGE™ 12 % Bis-Tris Protein Gel, 1.0 mm, NuPAGE™ 10x MOPS running buffer, NuPAGE® reducing agent, NuPAGE®20x MES running buffer were obtained from Thermo Fisher Scientific (Massachusetts, USA). Sequencing grade trypsin used for protein identification was obtained from Roche (Mannheim, Germany). Cornings® BioCoat™ poly-L-lysine coated plates were purchased from Corning (New York, USA). Coomassie brilliant blue-R250 was obtained from Biorad (California, USA). All other chemicals were obtained from Sigma Aldrich (Missouri, USA).

2.2. Sample preparation and heating treatment

The fresh goat raw milk was delivered at 4 °C then was centrifuged at 1500xg for 30 mins (Avanti Centrifuge J-26 XP, Beckman Coulter, USA) to remove the fat. The milk was then heated at 65, 70, 75, 80 and 85 °C for 30 mins in a water bath (Julabo SW23, Boven-Leeuwen, Nederland). The samples in each heating group were prepared in triplicate. The samples were put in ice water directly after heating to be cooled down to ambient temperature.

2.3. In vitro digestion

Simulated infant in vitro digestion was conducted according to Menard et al. (Menard et al., 2018), which includes a gastric phase and intestinal digestion phase. Five ml of raw or heated milk was added in each tube and preheated for 15 mins at 37 °C. For gastric phase, the pH value was adjusted to 5.3 by 1 M HCl and the ratio of goat milk to SGF was 63:37. The enzyme in gastric phase was pepsin with a working concentration of 268 U/ml. The whole process lasted for 60 mins and samples were collected at 0 time point (without enzyme), 10 and 60 mins. At each sampling time point, the pH value was adjusted to 6.6 by 1 M NaOH. Pancreatin used as intestinal enzyme was added according to the working concentration of trypsin with 16 U/mL and CaCl2 solution was added using a 3 mM working concentration. Five ml gastric chyme was transferred to intestinal phase digestion. After digestion for 0 (without enzyme), 10 and 60 mins, samples were taken and enzyme activity was stopped by 0.1 M Pefabloc (working concentration). Because the fat content in the skimmed milk samples was <1 %, lipase was not used. Every digestion sample was prepared in duplicate and all tubes were shaken at 37 °C in a water bath during the gastric and intestinal digestion process. After digestion, the samples were stored at −20 °C until further analyses.

2.4. Protein concentration determination

The samples after gastric or intestinal digestion were centrifuged at 4700 × g for 20 mins (Avanti Centrifuge J-26 XP, Beckman Coulter, USA) after which the supernatant was collected. The clots were freeze dried and stored at −20 °C. 200 μL supernatant was weighed in tin cups and dried in the incubator at 60 °C for at least 6 h. Each sample was prepared in duplicate. Then each tin cup was formed into a ball and protein content was detected by DUMAS Flash EA 1112 Protein analyser (Thermo Fisher Scientific, Massachusetts, USA). Nitrogen content was converted to protein content using a conversion factor of 6.38.

2.5. SDS-PAGE analysis

To investigate the protein composition of the main proteins, the supernatant and clots were both analyzed by SDS-PAGE gel. Gastric supernatants samples were diluted to the same protein concentration as the intestinal supernatant samples, after which the same volume of sample was loaded on the gel. The clots were freeze dried and 0.31–0.33 mg were weighed by a microbalance (Mettler Toledo, Columbus, USA). Samples were dissolved in 25 μL NuPAGE LDS sample buffer (4x concentrated, Invitrogen, USA), 10 μL NuPAGE sample reducing agent (10x concentrated, Invitrogen, USA), and 65 μL Milli-Q water. This was then centrifuged at 2000 rpm for 1 min at room temperature (Avanti Centrifuge J-26 XP, Beckman Coulter, USA) and then heated in a SW22 water bath (Julabo, USA) for 10 min. Samples were centrifuged again at the same condition. Finally, 12 % polyacrylamide NuPAGE bis-Tris 12-well gels were used. The following steps were carried out as described on previous studies (Xiong, Li, Boeren, Vervoort, & Hettinga, 2020).
2.6. Identification of the bands on the gel

LC-MS/MS was applied to identify some of the unknown bands visible on the SDS-PAGE gels of the gastric clots samples. After the digestion and centrifugation, the clots were dissolved in loading buffer to run on the gel. 12 bands on the gel were selected for identification by LC-MS/MS. For this, the gel was put in 25 ml 50 mM NH₄HCO₃ solution with 15 mM DTT and gently shaken for 1 h. Rinsed the gel then gently shook for 0.5 h at room temperature in 22.5 ml water with 1 ml pH = 8.1 M Tris and 20 mM Acrylamide in the dark. The 8 pieces of 1 mm² gel were cut from each band and transferred to 0.5 ml low binding micro centrifuge tubes (Avanti Centrifuge J-26 XP, Beckman Coulter, USA). Trypsin was added with a working concentration of 5 ng/µL and incubated overnight while shaking at room temperature (20 °C). Then, 10% TFA was used to adjust the samples’ pH value to 3 and all samples were filtered through a double C8-Filter into a 0.5 ml low binding Eppendorf. The filters were washed with 15 µL 50 % 1 ml/L HCOOH into the same Eppendorf tube. Most water was removed in the centrifugal vacuum concentrator (Z236202, Eppendorf, Germany) until a volume of 10–15 µL was reached. The final volume was adjusted to 50 µL with 1 ml/L HCOOH after which the samples were stored in the freezer (−20 °C) before injection onto Thermo nLC 1000 system (Thermo, Waltham, USA) with LTQ orbitrap XL (Thermo Fisher Scientific, Breda, The Netherlands). The detailed parameters of the instrument were described by Zenker et al. (2019). LC-MS/MS was processed using the MaxQuant version 1.6.3.4. For protein quantification, variable modifications included oxidation of methionine, deamidation of asparagine and glutamine, and acetylation of the peptide N-terminus. Peptides with length from 8 amino acids to 25 amino acids were identified by MaxQuant, with unspecific enzyme cleavage. Peptides were identified by the goat database UP000291000_2021 from Uniprot (downloaded in August 2021 from https://www.uniprot.org). Both peptide and protein false discovery rates were set to 1%. Main search peptide tolerance was 4.5 ppm.

2.10. Statistical analysis

Data analysis was performed by using GraphPad Prism 8 (GraphPad software, Inc.). The differences between the unheated group and the different heated groups were analyzed through one-way ANOVA. The protein content and degree of hydrolysis data are presented as the mean ± SEM (n = 2 or 3 per group) of each group. A p value < 0.05 was used to indicate a significant difference. Peptidomics-related data analysis was conducted using two online tools. Functional peptides were blasted by Milk Bioactive Peptide Database (August 2021 from http://mbpdb.unr.edu) and the Peptide alignment was studied by Peptigram (October 2021 from http://bioware.ucd.ie/peptigram/). The majority of the bioactive peptides in the database are derived from the major milk proteins: 36 % (338) β-casein, 13 % (119) αS₁-casein, 11 % (105) β-lactoglobulin, 10 % (98) κ-casein, 8 % (77) αS₂-casein, and 5 % (43) α-lactalbumin (Nielsen et al. 2017).

3. Results

3.1. The effects of heating on protein concentration of the digestion supernatant

The soluble nitrogen concentration in digestion supernatants was determined by the DUMAS method to evaluate the changes of soluble nitrogen during the digestion. The protein concentration was calculated by the results of nitrogen concentration and showed in Fig. 1. At G0, the protein concentration in all heated samples was lower compared to the non-heated sample. The decrease in protein concentration during gastric digestion can be attributed to the formation of the gastric clot. During intestinal digestion, proteins in the clots redissolved into the supernatant, as shown by the significant increase in protein concentration. At the end of intestinal digestion, all samples reached a similar level, which was close to the original protein concentration of the goat milk sample used.

At G0, the protein concentration of the unheated samples was highest whereas the protein concentration of the heated samples increased faster during the intestinal phase and gradually equalled the unheated control as digestion proceeded to 160. Compared to unheated control, samples heated at 65 and 70 °C showed a significantly higher protein concentration at 600. The protein concentration differed more between G0 and 160 as the temperature increased to 85 °C. The highest average protein concentration at 110 and 160 was observed in samples heated at 75 °C, with 33.0 ± 0.8 mg/ml and 36.1 ± 0.7 mg/ml, respectively.
3.2. Influence of heating on protein hydrolysis during the digestion process

Protein hydrolysis was determined based on the concentration of free amino groups (Fig. 2). There was only a minor increase in degree of hydrolysis during gastric digestion compared to G0 while intestinal digestion increased the degree of hydrolysis with about 36%. At the end of the intestinal phase, all the samples reached nearly the same level, ranging from 43 to 46%.

As for the effects of heating on hydrolysis, the degree of protein hydrolysis in control and heated samples showed significant differences only in the gastric phase. Values were significantly lower after heating and there was a trend that, except for the samples heated at 85°C at G60, the higher the heating temperature, the lower the degree of hydrolysis. For the intestinal phase, no significant differences were observed among all groups.

3.3. Main protein changes during digestion with various heating conditions

The protein changes in the supernatant and gastric clots after digestion were studied both by SDS-PAGE (Fig. 3B and C) and HPLC. Using HPLC, the intensities of the main proteins were individually analysed (Supplementary Fig. 1). The amount of clots at the bottom of the tubes decreased as the digestion proceeded from the gastric phase to the intestinal phase (Fig. 3A). Also, with increasing heating temperature, more precipitation could be observed at the bottom. The gastric clot was freeze dried and dissolved in loading buffer to run on the SDS-PAGE gel, after which 12 bands on the gel were excised after which proteins and large peptides were identified by LC-MS/MS.

3.3.1. Changes of main proteins in the supernatant after digestion

After gastric digestion, the caseins were digested into peptides with smaller molecular weight while whey proteins were still intact (Fig. 3B). After intestinal digestion, only whey proteins and digestion enzymes could be observed on the gel and the bands of whey proteins were lighter.
Fig. 3. A) Tubes without enzyme (G0) and after gastric digestion for 10, 60 min (G10, G60) and intestinal digestion for 10 and 60 min (I10, I60). B) Protein composition in the supernatant by SDS-PAGE. C) Protein composition in the gastric clots and intestinal precipitate by SDS-PAGE. D) Identification of protein bands from the SDS-PAGE gel by LC-MS/MS. NC indicates non-heated control samples. NC, non-heated control samples; 65–85 °C, samples after heating at 65–85 °C for 30 min.
as the heating temperature was higher. The β-LG band totally disappeared at I60 in the heated samples, while α-LA still showed a light band when heated below 80 °C. With heating temperatures higher than 80 °C, most intact protein bands, like LF and α-LA, disappeared.

The peak areas in the HPLC chromatogram were integrated, the results of which are shown in Supplementary Fig. 1, which are consistent with SDS-PAGE results. After gastric digestion for 10 min, the intensity of most intact proteins showed a sharp decrease. No casein was detected after gastric digestion, while α-LA and β-LG were still present at G10 and G60.

### 3.3.2. Changes in the main proteins in the gastric clots after digestion

To evaluate the digestion process more accurately, the gastric clots after digestion were studied for their protein composition by SDS-PAGE (Fig. 3C). The influence of heating on the gastric clot composition can be interpreted from the bands at the G0 time point, showing that more whey protein aggregated and became integrated in the gastric clots as the heating temperature increased. κ-casein was digested fastest, as it had totally disappeared after 10 min of gastric digestion, while αS2-casein was the most stable during gastric digestion. After gastric digestion, the caseins seemed to be cleaved into large peptides like bands 1, 2, 4, and 5 (Fig. 3C). After intestinal digestion these completely disappeared, indicating that they were digested into small peptides and free amino acids.

Band 3 and 6 in Fig. 3C are β-LG and α-LA. As gastric digestion proceeded, the relative intensity of whey proteins in the clots increased, indicating that they digested slower than caseins in the clot. The samples digested for 10 min in the intestinal phase showed darker bands in non-heated, 80 and 85 °C groups, while there was nearly no band visible at I10 in mild temperature heating groups (65, 70, and 75 °C).

### 3.3.3. Identification of proteins in the gastric clots

The SDS-PAGE gel of the gastric clots in samples heated at 70 and 80 °C was selected to identify the proteins present in different bands by LC-MS/MS. Caseins formed the main part of clots, with some whey protein aggregates (Fig. 3D). The main protein in band 1 was αS2-casein (85 % of total signal intensity) and in band 3 was αS1-casein (53 %). Other bands showed mixed protein composition but the proteins of the clots mainly included αS2-casein, β-casein, αS1-casein, κ-casein, β-LG, and α-LA. The complete protein composition of the 12 bands are shown in Supplementary Table 1.

### 3.4. Heat treatment-dependent differences in the peptide profiles

#### 3.4.1. LFQ intensity of peptides from main proteins

Peptidomics was performed on the supernatant of the digesta. In total, this resulted in 2562 different peptides being detected, that could be assigned to the major goat milk proteins. Fig. 4A shows that 30 % of the peptides originated from β-casein and 13 % of the peptides originated from κ-casein. αS1-casein and αS2-casein together accounted for 19 % of the peptides. The LFQ intensity values of samples after gastric and intestinal digestion for 60 min are shown in Fig. 4B. For the G60 samples heated at 85 °C, β-casein intensity is 35 % higher than non-heated samples, while β-LG is 6 %. After intestinal digestion for 60 min, the intensity of κ-casein and β-casein in the 85 °C treated samples had an abundance 47 % and 37 % higher than control samples, respectively. On top of that, for whey proteins, β-LG intensity in 85 °C heated sample showed a reverse trend with a decrease of 55 % while α-LA showed 50 % higher than control samples.

#### 3.4.2. Sequence alignment of the peptides within the main proteins

The peptide profiles of β-casein, κ-casein, αS1-casein, αS2-casein, α-LA, and β-LG were organized by the tool peptigram to visualize the peptide distribution and compare their intensities (Fig. 5A). The sequence coverage percentage for each protein is shown in Supplementary Fig. 2. The 65 °C heated samples showed higher intensities of peptides that are derived from specific regions of the proteins. For example for caseins, positions 180–200 of β-casein, 60–80 of κ-casein, 30–40 of αS1-casein showed major differences at G60, whereas for whey proteins, positions 110–120 of α-LA and 60–70 of β-LG showed major differences at I60.

#### 3.4.3. Bioactive peptides changes after the digestion and heat treatment

To compare the bioactivity of peptides of differently heated samples, all the peptides sequences were blasted with the MPBPD goat protein database and 17 bioactive peptides were found, as shown in Table 1. The intensities of those peptides after gastric or intestinal digestion are shown in Fig. 4B. Most bioactive peptides originated from β-casein and are related to antimicrobial function. After the gastric digestion of the non-heated sample, the intensity of antimicrobial peptides significantly increased but after heating the intensity of several peptides with bio-functionality declined, with 75 °C showing the lowest level. The peptides MHQPQPQL, LVYPFTGIPIN, GVPKVETMVPK, and VEELKPT-PEGNLE only appeared after the intestinal digestion phase, so the
Fig. 5. A) Peptigram profile of β-casein, κ-casein, αS1-casein, αS2-casein, α-LA, and β-LG. The color of the green bars is proportional to the sum of the peptide intensities overlapping at this position, while its height is proportional to the count of peptides overlapping. B) Heat map of the LFQ intensity of bioactive peptides. The data was log10 transformed and grey means that no intensity was detected. NC, non-heated control; 65–85 °C, samples after heating at 65–85 °C for 30 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
corresponding ACE-inhibitory and DPP-IV inhibitory function may only appear intestinally. The samples heated at 65 °C showed the highest functional peptide intensities at both I10 and I60 (Supplementary Fig. 5). Compared to the unheated control samples, the intensity of ACE-inhibitory and antimicrobial peptides showed sharp increase. The 75 and 85 °C heated samples showed a higher level of total functional peptides compared to the control samples at the end of the intestinal digestion.

4. Discussion

Goat milk has recently attracted increasing attention, especially regarding its use in infant formula. The object of this research was to analyze the effects of heating on skimmed goat milk proteins and explore the digestibility changes in an in vitro infant digestion model.

From DUMAS, SDS-PAGE, and HPLC analyses of the supernatant (Fig. 1, Fig. 3B, Supplementary Fig. 1), the results consistently showed that the supernatant protein concentration of the heated samples was significantly decreased at 60 especially at 85 °C. HPLC results showed that this is mainly due to the disappearance of \( \kappa \)-casein, \( \alpha_\text{S2}-\)casein, and \( \alpha\text{-LA} \). This implies that the 85 °C heated samples had lower supernatant protein concentration but larger amounts of protein in the gastric clots, probably due to increased whey protein-casein interactions after the heating step. Even though this sample started with lower supernatant protein concentration, it showed a similar final value (I10 and I60) of heating step. Even though this sample started with lower supernatant protein concentration, it showed a similar final value (I10 and I60) of heating step. Even though this sample started with lower supernatant protein concentration, it showed a similar final value (I10 and I60) of heating step. Even though this sample started with lower supernatant protein concentration, it showed a similar final value (I10 and I60) of heating step. Even though this sample started with lower supernatant protein concentration, it showed a similar final value (I10 and I60) of heating step. Even though this sample started with lower supernatant protein concentration, it showed a similar final value (I10 and I60)

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**Table 1**

Peptide sequence, protein of origin, and described bioactive function according to the MBPDB of the detected bioactive peptides. \[ \text{Table 1. Peptide sequence, protein of origin, and described bioactive function according to the MBPDB of the detected bioactive peptides.} \]

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Protein of origin</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>INNQLFIPPIYY</td>
<td>( \kappa )-casein (72-81)</td>
<td>DPP-IV Inhibitory</td>
</tr>
<tr>
<td>AASETPVHIH</td>
<td>( \kappa )-casein (147-154)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>GLDQKEVAGT</td>
<td>( \beta )-lactoglobulin (27-36)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>LAFNPIQLEOG</td>
<td>( \beta )-lactoglobulin (167-177)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>MAIPPRKDKQTTEYPAINT</td>
<td>( \kappa )-casein (127-145)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>MHQPPQPL</td>
<td>( \beta )-casein (159-166)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>PFGTPGPNLPOQ</td>
<td>( \beta )-casein (76-87)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>FYHISSTPTE</td>
<td>( \kappa )-casein (151-160)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>PVVVPFQFLPE</td>
<td>( \beta )-casein (96-106)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>REQELN</td>
<td>( \beta )-casein (16-23)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>TEDLEQDKHF</td>
<td>( \beta )-casein (56-67)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>TPEFDEKEAL</td>
<td>( \beta )-lactoglobulin (143-152)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>VEEELPTPEGNLIE</td>
<td>( \beta )-lactoglobulin (61-73)</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>YQRPVLGVPGERPFI</td>
<td>( \beta )-casein (206-220)</td>
<td>Antimicrobial</td>
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<td>GVPVKETMVPK</td>
<td>( \beta )-casein (109-120)</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td>GVPVKETMVPKHI</td>
<td>( \beta )-casein (109-121)</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td>LLYFPTGPINP</td>
<td>( \beta )-casein (73-83)</td>
<td>ACE-inhibitory</td>
</tr>
</tbody>
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Heating with high temperatures (\( \geq 80 \) °C) showed a large influence on the soluble protein concentration whether before or after digestion, whereas lower temperatures (\( \leq 70 \) °C) heating showed relatively slight effects. At G10 and G60, protein concentration in the supernatant of samples heated at 65 °C was higher than of control samples, whereas samples heated at higher temperatures showed lower values (Fig. 1). The same trend can also be observed from intact protein intensities by HPLC (Supplementary Fig. 1), which also showed that whey proteins were the main reason for this increase in the supernatant. This indicates that at lower heating temperature, more whey proteins remain present as soluble protein in the supernatant. SDS-PAGE of the intestinal clots (Fig. 3C) showed that proteins in mildly heated samples were completely digested. In samples that were heated at higher temperature, whey proteins bands seemed more intense at I10 and I60. Heating for 30 min at 80 °C seems to be the threshold above which whey proteins are denatured and aggregated to such a degree that they are not completely digested in the clot. This may be explained by the increased formation of aggregates after high temperature heating. These large and compact whey protein aggregates may hamper whey protein digestion in these high temperature treated samples (Sah et al. 2016). In conclusion, low temperature heating promoted clot digestion, while higher heating temperatures showed higher clots digestion efficiency. However, at the end of the digestion, the clots showed a larger amount of undisgested whey proteins due to severe aggregation.

Compared to cow milk, many studies stated that goat milk showed a lower extent of gastric coagulation, higher hydrolysis degree and goat milk proteins were hydrolyzed faster (Hodgkinson et al., 2018; Roy, Ye, Moughan, & Singh, 2020). Jasińska (1995) compared the degree of hydrolysis during pepsin and trypsin hydrolysis, which for cow milk proteins was 23–42 % and 76–90 %, while for goat milk proteins it was 65 % and 96 %, respectively. Another in vitro study reported that goat milk proteins showed a lower extent of coagulation, and different protein hydrolysis kinetics compared to cow milk proteins, with goat milk proteins being hydrolyzed faster at the start of digestion (Maathuis, Havenaar, He, & Bellmann, 2017). The possible reason for these differences may be that goat milk formed smaller gastric clots and these differences of clot structure may play a role in digestion kinetics (Roy, Ye, Moughan, & Singh, 2020). A different composition of the goat milk casein fraction, especially a lower level of \( \alpha_\text{S1-CN} \), which give rise to larger casein micelle with more hydrated pores, may be an underlying factor for this difference in gastric clotting behaviour (Clark & Sherbon, 2000, Prosser, 2021). Our results (Fig. 2) showed that samples after heating had a lower level of gastric protein hydrolysis which decreased steadily as the temperature increased. This may be due to the strong decrease in protein concentration in the supernatant, which resulted in less protein with less free amino groups released during digestion. In summary, protein aggregation seems to be the predominant factor inducing a decreased gastric protein hydrolysis rate after more intense heating.

\( \kappa \)-casein is a major source of bioactive peptides in this study (Table 1), which is similar to cow milk (Boutrou, Henry, & Sanchez-Rivera, 2015). This can be explained by both the high abundance of \( \beta \)-casein in goat milk and its high susceptibility to proteolysis. High temperature heating reduced the level of bioactive peptides, whereas the samples heated at 65 °C showed a relatively higher level of bioactive peptides compared with the other samples. For heating temperatures
above 75 °C, lower levels of bioactive peptides were found at the end of intestinal digestion. The peptides with similar sequence as bioactive peptides were selected, and their intensities were shown in Supplementary Fig. 4. Compared to the bioactive peptides in the database, the peptides with shorter sequence were defined as truncated fractions and longer as precursors. The high temperature heated samples showed a higher intensity of the truncated form of bioactive peptides, while the 65 °C heated samples showed higher intensity of the precursors of bioactive peptides. This indicates that in the high temperature treated samples, the bioactive peptides were further broken down during digestion. This enhanced breakdown at higher temperature may be due to more extensive enzyme cleavage of these samples, which is consistent with the higher digestion rate and easier cleavage digestion seen in the samples heated at 85 °C.

5. Conclusion

Heating goat milk proteins at different temperatures, aiming at different levels of protein denaturation, led to changes in protein digestion mechanisms during in vitro infant digestion. High temperature heating (≥80 °C) induced more extensive clot formation, due to increased whey protein-casein interaction, resulting in a higher digestion rate of clots. Although casein digestion rate hardly changed with heating temperature, whey protein digestion depended on heating temperature. High temperature heating induced reduced whey protein digestion, due to extensive whey protein aggregation, whereas the lowest heating temperatures, reduced the whey protein digestion in the supernatant, due to its compact native state. Digestion of 65 °C heated samples resulted in a higher intensity of bioactive peptides compared to other heat treatments, whereas heating above 75 °C induced further cleavage of bioactive peptides, resulting in lower intensities of them.

CRediT authorship contribution statement

Qing Ren: Conceptualization, Formal analysis, Methodology, Software, Visualization, Writing – original draft. Mattia Boiani: Supervision, Writing – review & editing. Tao He: Supervision, Writing – review & editing. Harry J. Wichers: Supervision, Writing – review & editing. Kasper A. Hettinga: Conceptualization, Software, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The author declares the following financial interests/personal relationships which may be considered as potential competing interests: Kasper A. Hettinga reports financial support was provided by Ausnutria B.V.

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

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References


