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Digestibility of Transglutaminase Cross-Linked Caseinate versus Native Caseinate in an In Vitro Multicompartmental Model Simulating Young Child and Adult Gastrointestinal Conditions

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ABSTRACT: Aim of this study was to investigate the digestion of transglutaminase cross-linked caseinate (XLC) versus native caseinate (NC) in solution and in cheese spread under digestive conditions for adults and children mimicked in a gastrointestinal model. Samples were collected for gel electrophoresis and nitrogen analysis. The results showed no relevant differences between XLC and NC for total and α-amino nitrogen in digested fraction under adult and child conditions. However, the rate of digestion was depending on the food matrix. Gel electrophoresis showed the gastric breakdown of XLC without formation of pepsin resistant peptides larger than 4 kDa. NC was slowly digested in the stomach with formation of pepsin resistant fragments and was still detectable in the stomach after 90 min. In the small intestine the proteins were rapidly digested. XLC was digested to small peptides, while NC was resistant against pepsin digestion under gastric conditions of adults and children.

KEYWORDS: *Cross-linked caseinate, food matrix, in vitro digestion, adults and children*

INTRODUCTION

Microbial transglutaminase (Tgase) catalyzes in vitro cross-linking of food proteins, such as soy, wheat, whey proteins, and casein.[1] Compared to native caseinate (NC), cross-linked caseinate (XLC) has specific physical properties that are beneficial in food applications.[2] Depending on the intensity of the cross-linking by Tgase, XLC has technological advantages over native NC in terms of water-holding capacity, viscosity, and viscoelasticity in food products,[3,4] whether or not cross-linked in combination with gelatin.[5] These improved physical properties can be used in food processing, such as 15% increased water holding capacity in yoghurt[6] and reduced syneresis in cheese.[7] For example in cheese spread as used in this study, 30% less sodium caseinate can be used when it is partially Tgase cross-linked to reach the same viscosity as with native sodium caseinate.

However, information about the digestibility of Tgase XLC in comparison to NC is scarce, while digestibility might have serious implications for the nutritional quality of the proteins as well as the risk for food allergy. A static in vitro pepsin digestion study indicated a similar or maybe even better rate of digestion for Tgase XLC versus NC.[8] Monogioudi et al.,[9] on the contrary, showed that Tgase and tyrosinase (TrTyr) XLC was more stable for pepsin digestion than NC. Monogioudi[10] tested "almost completely cross-linked" casein by Tgase and TrTyr and concluded that XLC was more resistant to digestion under in vitro acidic gastric conditions than NC. So, the degree of cross-linking may play a role in the rate of digestion. Also the food matrix may influence the digestibility of potential food allergens, such as NC.[11] In other words, it is crucial to determine the digestibility of XLC exactly as it is used in the food product during passage through the stomach and first part of the small intestine in comparison to NC.

Caseinate-containing products are consumed by adults as well as by children of 6 months of age and older. The gastrointestinal conditions, however, are different between these young age groups and adults.[12−14] Therefore, the digestibility should be investigated for adults as well as children of 6−18 months. Testing in a clinical study, specifically in children, is difficult and has serious ethical drawbacks. A good alternative is to study protein digestion in in vitro systems. As indicated by Moreno[15] and Wickham et al.,[16] this should preferably be done in physiologically relevant, dynamic models, simulating gastrointestinal transit of the food and digestive processes in the stomach and small intestine.

The TNO in vitro gastrointestinal model (TIM-1 system) is such a dynamic system.[17] In TIM-1 the conditions can be simulated as in the lumen of the upper gastrointestinal tract in relation to the age group (infants, adults, elderly), the type and amount of meal and health conditions. The TIM system has been validated for the digestion and availability for absorption of various nutrients in comparison with in vivo studies. This include the digestibility of proteins[18,19] and fats[20,21] as well as water-soluble vitamins,[22,23] fat soluble vitamins,[24,25] minerals,[26,27] bioactive compounds,[28,29] and functional proteins[30] and immuno-reactive proteins in combination with gel electrophoresis and T-cell bioassays.[31] For protein digestion studies the slightly simplified Tiny-TIM system (Figure 1) has been validated for the digestion of proteins and the bioaccessibility of amino acids.[32,33]

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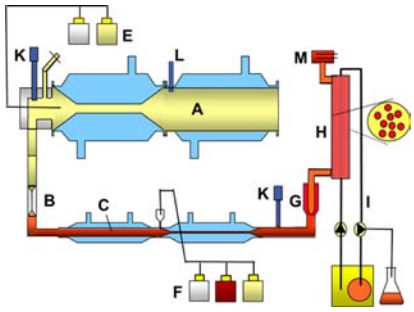

Figure 1. Tiny-TIM system. A. gastric compartment with flexible wall inside for peristaltic movements; water at 37 °C around it for the body temperature; B. pyloric sphincter for controlled gastric emptying; C. small-intestinal compartment with flexible wall; E. secretion of gastric acid, salivary and gastric enzymes and electrolytes; F. intestinal secretion of bile, pancreatic juice and bicarbonate; G. prefilter; H. dialysis via hollow-fiber semipermeable membrane; I. collection of dialysis fluid and water absorption; K. pH electrodes; L. pressure sensor; M. level sensor.

This is the first study simulating the dynamic gastrointestinal conditions for adults and young children to investigate the degree of gastric and intestinal digestion of Tgase XLC in comparison to NC, with and without a realistic meal matrix. These types of studies might help to evaluate the risk of sensitization to these proteins and the development of food allergy.

MATERIALS AND METHODS

Test Products. Tgase cross-linked bovine sodium caseinate Excellion EM7 High Viscosity (XLC), native sodium caseinate (NC), cheese spread containing 5.6% cross-linked sodium caseinate (cheese spread XLC), and cheese spread containing 8% native sodium caseinate (cheese spread NC) were kindly provided by FrieslandCampina DMV (Veghel, The Netherlands). The cheese spreads were freshly prepared one day before use in the TIM system. The composition of the cheese spreads is given in Table 1 (information from FrieslandCampina DMV).

Gastro-Intestinal Model. The experiments were performed in the Tiny-TIM system (Figure 1) comprising compartments for the stomach, pyloric sphincter, and small intestine.[17,32] These compartments had an internal flexible wall for peristaltic mixing and controlling gastric emptying. The gastrointestinal content was kept at body temperature (37 ± 0.5 °C) and mixed with the secretion fluids: saliva with electrolytes and amylase; gastric juice with pepsin, lipase and gastric acid; pancreatic juice with bicarbonate and digestive enzymes (proteases, lipase, amylase); and bile. All at physiologically relevant concentrations and activities at site specific pH levels. Connected to the compartment of the small intestine there was a unit with hollow-fiber semipermeable membranes with a cutoff of 5−7 kDa and a surface area for absorption of 0.7 m^2 (Sureflux 70L^{GA}, Nipro Europe, Belgium). This unit allowed the dialysis of digested and dissolved nutrients, such as small peptides and amino acids, and the absorption of water. The dialyzed nutrients represent the compounds that are available for intestinal absorption (bioaccessible fraction) during digestion, e.g. small peptides and amino acids analyzed as the amount of total nitrogen and α-amino nitrogen. All parameters and settings were continuously computer-monitored and -controlled with specifically developed software.

Table 1. Ingredients and Macro-Nutrients Analysis in the Cheese Spread Containing XLC and the Cheese Spread Containing NC

cheese spread	XLC	NC
Ingredients	%	%
melting salt	2	2
gouda cheese 48+	44.9	44.9
butter	14	14
SMP	3	3
whey powder	2.4	0
cross-linked caseinate	5.6	0
native caseinate (EM7)	0	8.0
water/condensate	28.1	28.1
Analysis		
% dry matter	53.0	53.0
% fat (as is)	25.5	25.5
% protein (as is)	16.7	18.7
% lactose (as is)	3.2	1.5

Simulated Gastrointestinal Conditions. The test products were investigated in the Tiny-TIM system simulating the average conditions in the stomach and small intestine of young children (6−18 months) and healthy adults after the intake of a light meal. The TIM settings for physiological parameters, such as gastric pH, gastric emptying, peristalsis, and the composition and amount of the secretion fluids are based on a broad literature search on physiological data of adults and infants.[12−14,17,18] Artificial oral fluid with α-amylase (A6380-1G, Sigma-Aldrich) and gastric juice with gastric lipase (F-AP 15, Amano, Nagoya, Japan) and pepsin (P7012-10G, Sigma-Aldrich) were gradually added into the gastric compartment.[17,18] Bile (P8631−100G, Sigma-Aldrich), pancreatin (Pancrex-Vet, Pfizer, Karlsruhe, Germany) purified by centrifugation and electrolytes were gradually added to the duodenal compartment.[17,18] The composition and amount of secretion fluids simulate the physiological levels after the intake of a meal.

The major difference in the TIM-settings between young child and adult conditions is the gastric pH. In young children the gastric pH drops from 6.5 to 4.5 in 150 min and in adults from pH 5.5 to 2.0 in 90 min and from pH 2.0 to 1.7 from 90 to 150 min. This difference in pH curve has consequences for the activity of pepsin in the breakdown of proteins. The secretion of digestive enzymes per kg body weight is not different between children and adults. Therefore they were standardized in this study, relative to the amount of protein intake (section below).

Set-Up of the TIM Experiments. Prior to the performance of each TIM experiment, the secretion fluids were freshly prepared, the pH electrodes calibrated, and new semipermeable membranes units installed. The intake of XLC and NC was standardized at 7 g of caseinate for the adult and young child conditions. This means a difference in downscaling between child and adult conditions (approximately factor 1.5 and 4, respectively) for the intake as well as the amount of secretion fluids and digestive enzyme activity. The two products were tested without a meal matrix. The caseinates were gradually added to the salivary/gastric juice as used in the TIM system under sturdy homogenization on a magnetic stirrer at 70 °C to solubilize the caseinate. The pH was set at 6.5.

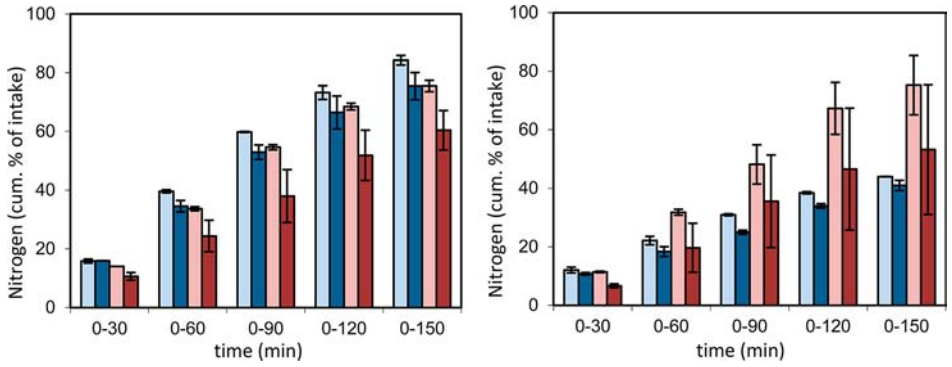

Figure 2. Mean (±range; $n = 2$) cumulative bioaccessible amount of nitrogen as percentage of nitrogen intake during digestion of XLC and NC in solution (light and dark blue bar, respectively) and XLC and NC in cheese spread on a slice of bread (light and dark red, respectively) under simulation of the gastrointestinal conditions of young children (left) and of adults (right).

The intake of XLC and NC cheese spread products was standardized at 2.25 g cross-linked or native caseinate (Table 1). Therefore, the amount of cheese spread added to the TIM system was 40 and 28 g for cheese spread with XLC and NC, respectively. As a consequence, the intake of protein from the spreads was different between these two products: 5.24 g protein for cheese spread XLC and 6.68 g protein for cheese spread NC. The products were tested with a "meal matrix": the cheese spread was spread on a slice of white milk-free wheat bread (25 g). The slice of bread contained 8% protein (2 g). The total protein intake was 7.24 and 8.68 g for the cheese spread XLC meal and cheese spread NC meal, respectively.

The secretion fluids contained approximately 0.5 g endogenous proteins (enzymes). This makes that the total protein "input" (= exogenous plus endogenous protein) was 7.50 g (1175 mg N_2) for both the XLC and NC, and 7.74 g (1210 mg N_2) and 9.18 g (1435 mg N_2) for the experiments with cheese spread XLC and cheese spread NC, respectively. This small difference in the total amount of protein input (1.44 g = 17%) will not influence the digestibility of proteins, due to excess of digestive enzymes in the gastro-intestinal tract. For determining the protein digestion, this difference in amount of nitrogen intake was corrected by calculating the bioaccessible amount of nitrogen as percentage of total nitrogen intake.

Sampling. During the experiments 3 mL samples were taken from the lumen of the stomach and small intestine compartments at 15 min intervals for 150 min. Immediately after collection, three 1 mL subsamples were snap frozen in dry ice, in order to stop the digestive activity. The samples were stored at −18 °C.

During the TIM experiments, dialysate samples were collected from the small intestine compartment in 30 min aliquots (0−30 min, 30−60 min etc.) for 150 min. The collected volume was measured and three subsamples of 10 mL were stored at −18 °C for nitrogen analysis.

Analysis. The dialysate samples were analyzed for total nitrogen (Kjeldahl) to calculate the ileal digestibility of protein during gastrointestinal passage in time as mean (±range; $n = 2$) absolute amount of bioaccessible nitrogen (mg) and as percentage of intake (to overcome differences in protein intake). The dialysate samples were also analyzed for α-amino-nitrogen to calculate NH_2-ending protein fragments as mean (±range; $n = 2$) absolute amount (mmol). The NH_2 groups were determined with 2,4,6-trinitrobenzen-sulfonic acid (TNBS) at $\lambda = 405$ nm and glycine as internal standard. Higher amounts of NH_2-ending proteins are an indication for the presence of higher numbers of peptides and therefore smaller peptides.

The lumen samples were analyzed for protein fragments of 6−200 kDa by gel electrophoresis (SDS-PAGE). Bradford analysis[34] was performed to determine the amount of protein for standardization of these samples on the amount of solubilized protein (15 μg). To 1 mL of gastric lumen samples, 100 μL of 1 M Tris pH 11 was added to neutralize the pH for pepsin inactivation and better solubility of casein. Duodenum lumen samples (pH 6.5) were directly used for Bradford analysis and electrophoresis. SDS-PAGE was performed using a BioRad Mini Protean II system (BioRad, Hercules, CA) with 15% acrylamide gels (15 × 10 cm). Prestained molecular weight markers with molecular weights of 6.5, 14.4, 20.1 30, 43, 67, 94, and 220 kDa were used as reference. Samples on ice were mixed in a 1 to 1 ratio with 63 mM TRIS buffer (pH 6.8) containing 1% dithiotreitol (DTT), 2% SDS, 0.01% bromophenol blue and 20% (v/v) glycerol and were subsequently boiled for 5 min (including inactivation of pancreatic enzymes). Proteins were stained by adding 2.5 mL 1% CBB R250 in ethanol to 50 mL 12.5% TCA that was used for fixation. Destaining was done with 12.5% TCA.

For analysis for casein immune reactive fragments, lumen samples were selected based on gastric digestion results and on expected high levels of caseinate or caseinate fragments emptied from the stomach. A standardized ELISA method was performed according to the kit instructions using Veratox^R Quantitative Allergen Test for casein allergens (Neogen Europe, Auchineruive, UK) with a detection limit of 2 ppm.

The Kjeldahl and alpha-amino nitrogen data were statistically analyzed with student t test using Excel software.

RESULTS

Protein Digestibility Under GI Conditions of Young Children and Adults. The cumulative bioaccessible amount of nitrogen in 30 min periods of digestion was similar when comparing XLC and NC given in a solution or in cheese spread ($p > 0.1$). There was a trend for a higher nitrogen bioaccessibility for XLC than for NC. This was found under the gastrointestinal conditions for young children (Figure 2, left) as well as for adults (Figure 2, right). Under young child conditions the total ileal protein digestibility after 150 min of the XLC and NC solution was 84 ± 2% and 75 ± 5% (p=0.2), respectively, and of the XLC and NC in cheese spread it was 75 ± 2% and 60 ± 7% (p=0.1), respectively (Figure 2, left). Under adult conditions the ileal protein digestibility of the XLC and

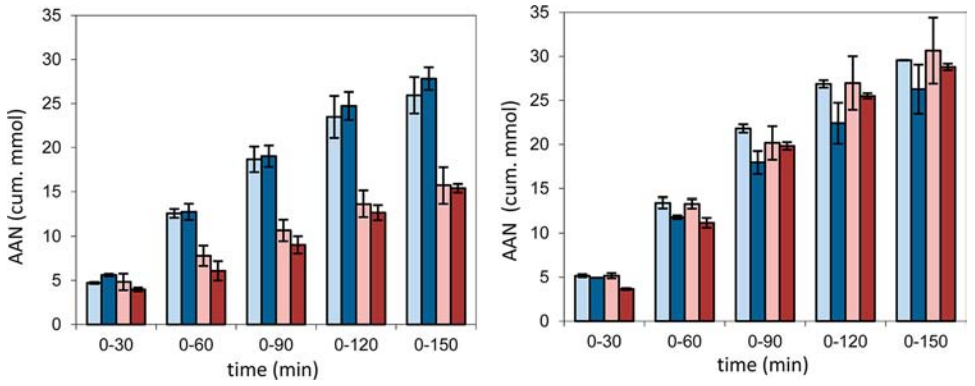

Figure 3. Mean (mmol ± range; $n = 2$) cumulative bioaccessible amount of alpha-amino nitrogen during digestion of XLC and NC in solution (light and dark blue bar, respectively) and XLC and NC in cheese spread on a slice of bread (light and dark red bar, respectively) under simulation of the gastrointestinal conditions of young children (left) and adults (right).

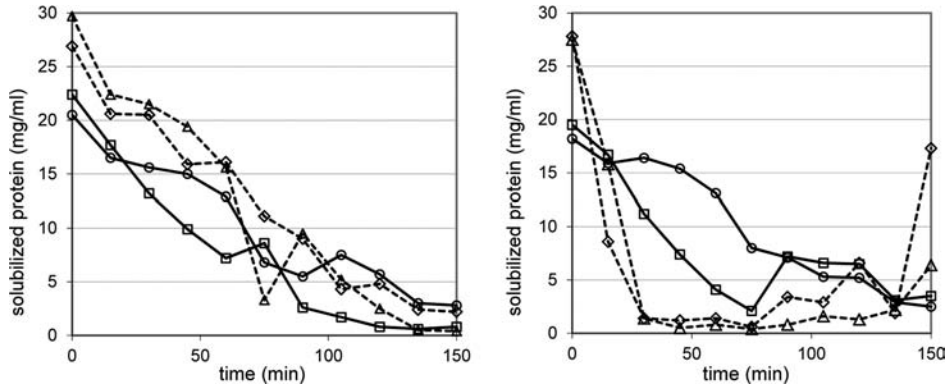

Figure 4. Solubilized protein concentration (mg/mL) in the lumen of the gastric compartment in time, analyzed by Bradford assay[34] for the experiments under gastrointestinal conditions of young children (left) and adults (right) with XLC and NC in solution (dotted lines) and in cheese spread (solid lines); XLC solution (Δ), NC solution (◊), XLC spread (○), NC spread (□).

NC solutions was 44 ± 1% and 41 ± 2% ($p = 0.2$), respectively and in the cheese spread products it was 75 ± 8% and 55 ± 15% ($p = 0.4$), respectively (Figure 2, right).

Under young child and adult gastrointestinal conditions (Figure 3), the bioaccessible amounts of α-amino-nitrogen were not significantly different between XLC and NC when given as solution or in cheese spread. However, α-amino-nitrogen was higher for both XLC and NC when given as solution than when given as cheese spread on a slice of bread ($p < 0.05$). This difference between the solution and the cheese spread was not found for the adults conditions.

Breakdown of XLC versus NC in the Stomach. The lumen samples for SDS-PAGE analysis were standardized on the amount of solubilized protein based on the Bradford assay. Under young child conditions the solubilized protein from NC and XLC solutions in the stomach gradually decreased from 27 mg/mL to 5 mg/mL in 120 min (Figure 4). In contrast, under adult conditions the concentrations in the gastric lumen of solubilized protein from these products decreased rapidly in time from 27 mg/mL to less than 3 mg/mL in 30 min, while it gradually increased again after 90 min. This was related to the visual observation of differences in the rate of coagulation of the casein during gastric pH decrease under adult versus young child gastric conditions, while after 150 min the coagulated protein solubilizes again. The concentrations of solubilized protein from NC and XLC in the cheese spread products decreased gradually in time from 20 mg/mL to 5 mg/mL in 120 min for both the adult and young child conditions. The concentration of solubilized protein in the content of the small intestine increased gradually in time from approximately 1.5 mg/mL at the start to 3.5 to 5.5 mg/mL after 120 min.

The results of the SDS-PAGE of XLC and NC solution in the gastric lumen samples taken from the Tiny-TIM system under gastrointestinal conditions of young children and adults are shown in Figures 5 and 6, respectively. The sample taken at the start of the XLC solution experiments (Figure 5, left, lane 1) show the presence of XLC (above 200 kDa), serum albumin (67 kDa), pepsin (~40 kDa), casein (25−32 kDa), and the

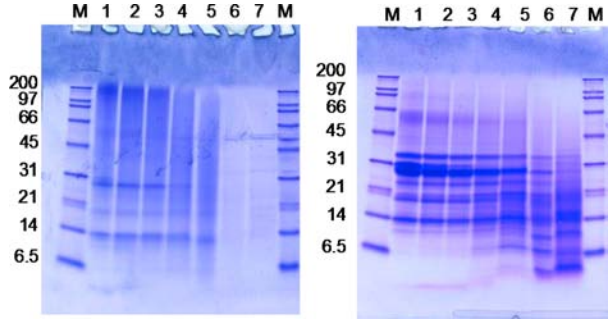

Figure 5. SDS-PAGE of gastric lumen samples (15 μg protein per lane) of XLC (left) and NC (right) in solution and tested under conditions of young children. Lane M = marker proteins, lanes 1−7 are lumen samples taken at 0, 15, 30, 60, 75, 90, and 105 min after intake.

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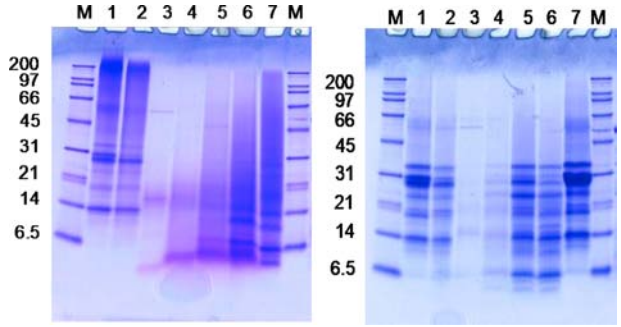

Figure 6. SDS-PAGE of gastric lumen samples (15 μg protein per lane) of XLC (left) and NC (right) in solution and tested under adult conditions. Lane M = marker proteins, lanes 1−7 are lumen samples taken at 0, 15, 30, 60, 75, 90, and 105 min after intake.

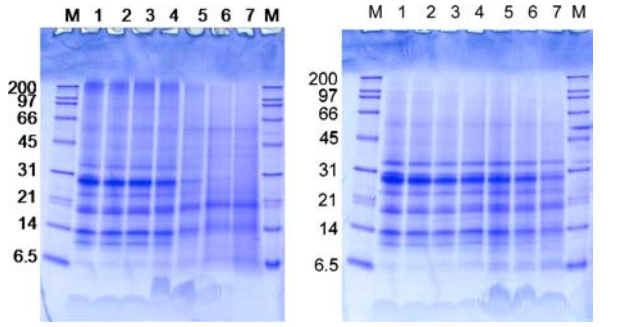

Figure 7. SDS-PAGE of gastric lumen samples (15 μg protein per lane) of XLC (left) and NC (right) in cheese spread tested under conditions of young children. Lane M = marker proteins, lanes 1−7 are lumen samples taken at 0, 15, 30, 60, 75, 90, and 105 min after intake.

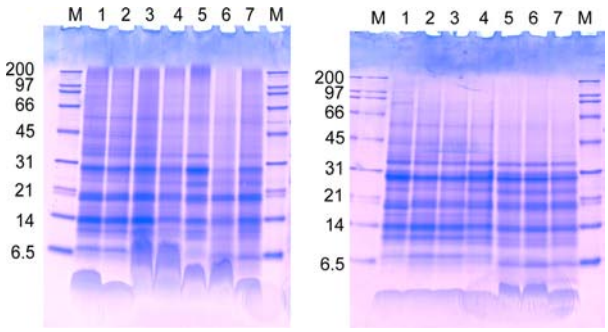

Figure 8. SDS-PAGE of gastric lumen samples (15 μg protein per lane) of XLC in cheese spread (left) and NC in cheese spread (right) tested under adult conditions. Lane M = marker proteins, lanes 1−7 are lumen samples taken at 0, 15, 30, 60, 75, 90, and 105 min after intake.

whey proteins ß-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa). The gastric lumen samples taken after 15 to 105 min (Figure 5, lanes 2 to 7) show the disappearance of the XLC and casein bands after 60−90 min, without showing clear new protein bands below the XLC or casein bands. This indicates the digestion of the XLC in the stomach. The samples taken at 90 and 105 min (lanes 6 and 7) hardly show any protein bands between 6.5 and 200 kDa, despite the fact that the samples were corrected for the protein content.

The samples taken at the start of the NC solution experiments (Figure 5, right, lane 1) showed the same protein bands as XLC, although the casein and whey protein bands were more intensive and no cross-linked casein was present. The casein band in the stomach samples was still present after 90−105 min under simulation of the gastrointestinal conditions of young children (Figure 5, lanes 6 and 7) as well as adults (Figure 6, lanes 6 and 7). The intensity of the casein band gradually decreased in time despite the standardization of the samples on the amount of protein. It also shows the appearance of bands of smaller molecular weight proteins (6−20 kDa) from 60 to 105 min (Figures 5 and 6, lanes 5−7).

The gastric samples of XLC and NC collected from the experiments under adult conditions show an unexpected pattern (Figure 6). The samples collected at the start and after 15 min (lanes 1 and 2) show the presence of relatively high levels of casein. In the time period between 30 and 90 min for XLC and between 30 and 60 min for NC no clear protein bands were detectable; indicating low levels of solubilized protein between 6 and 200 kDa. This corresponds with the Bradford protein analysis (Figure 4) showing a strong decrease in the concentration of solubilized proteins after 30 min. This relates to the visual observation of precipitation of caseinate in the stomach, due to the low gastric pH under adult conditions.

The SDS-PAGE analysis of gastric lumen samples collected from the experiments with cheese spread containing XLC or NC under gastrointestinal conditions of young children and adults are shown in Figures 7 and 8, respectively. The electrophoresis with cheese spread containing XLC showed, besides the presence of XLC (above 200 kDa), also relatively high amounts of casein (20−30 kDa) and whey proteins (18 and 14 kDa). Under young children conditions the time samples show the disappearance of the XLC band as well as the casein bands after 90 min (Figure 7, left, lanes 5−7), indicating the digestion of these proteins in the stomach. The lumen samples taken in time from the stomach did not show the appearance of new protein fragments between 6 and 20 kDa, but the whey protein bands did not completely disappear.

Under adult GI conditions the time samples also show the disappearance of the XLC band (Figure 8, left). The casein and whey protein bands were still quite intensive after 90 to 105 min (Figure 8, lanes 6 and 7) after the intake of cheese spread containing NC as well as XLC.

To detect the concentration of immune reactive casein proteins or casein fragment during the digestion process, selected gastric lumen samples from the cheese spread experiments under conditions of young children and adults were also analyzed in an ELISA assay using rabbit anticasein antibodies. The results show similar concentrations of immune reactive casein in the stomach compartment at 10 min after intake of the cheese spread containing XLC or NC (28.0−33.5 mg/mL) for both age groups. After 60 min the concentration in the stomach compartment was reduced to 2 to 8 mg/mL for both products and age groups.

Breakdown of XLC and NC in the Proximal Small Intestine. The samples collected from the small intestinal lumen in time (every 15 min for 135 min) only show the proteins from the pancreatin secretion fluid, among others pancreatic enzymes. Neither XLC nor NC bands are visible in the experiments with XLC and NC as solution and in cheese spread (figure not shown). Although there is a strong background by the pancreatic enzymes, if new digestion proteins were produced in the small intestine, new bands between 30 and 6 kDa would be visible, due to the high

reproducibility of the background proteins from the pancreatic secretion.

Immune Reactive Casein. Additional intestinal lumen samples collected at 30 min after intake were analyzed in an ELISA assay using rabbit anticasein antibodies. These time point samples should have the highest amount of caseinate emptied from the stomach into the proximal small intestine. The results show that the concentrations of immune reactive casein were below the minimal detection limit of 0.05 mg/mL for both products and both age groups.

DISCUSSION

Caseinate such as in yoghurt and cheese products can be cross-linked by transglutaminase for food technological advantages over native caseinate.[2−7] Dairy products are consumed by a large part of the population, especially in Western Europe, by adults as well as young children, because of its taste and source of amino acids, minerals and vitamins. On the other hand, casein is one of the milk proteins that are on the list of food allergens, with an incidence of 2−6% in children.[35] Milk protein allergy might partly be related to the delayed digestion of casein. For these two reasons it is crucial to have information on the digestibility of cross-linked caseinate (XLC) versus native caseinate (NC). Especially, because the use of cross-linked caseinate in food products is expected to increase due to newly reported beneficial properties, such as that it improves the oxidative stability of oil emulsions.[36] This is the first study under simulation of the dynamic gastrointestinal conditions for adults and young children investigating the digestibility of NC versus XLC in solution and in a realistic food matrix.

Due to difference in viscosity between de NC and XLC cheese spread products it might be expected that this influences the rate of gastric emptying. This was studied in ileum-fistulated mini-pigs with PEG-4000 as transit marker[37] and in a human study with ^{13}C-breath test as gastric emptying marker.[38] Both studies demonstrated that XLC and NC in solid and liquid products had the same gastric and intestinal transit times. So, in the TIM experiments we used the same settings for gastric emptying of the NC and XLC products.

During the experiments, the gastric pH gradually decreased according to preset points by controlled secretion of gastric acid. Visual observation showed a clear coagulation of the casein in the gastric compartment starting at pH below 5, which was reached within 15 min under adult gastric conditions. This corresponds with the Bradford analysis for the solubilized proteins, showing a drop in the concentration from 27 mg/mL to 3 mg/mL within 30 min after intake. Under simulation of the GI conditions of young children (6−18 months of age) the gastric pH drops from 6.5 to 4.5 in 150 min. Consequently, there was a delayed visual coagulation of NC and XLC, corresponding with the more gradual drop in concentrations of solubilized protein according to Bradford analysis. No difference was found between NC and XLC in the rate of coagulation due to gastric acid. This might be different for rennet coagulation. Bönisch et al.[39] described an increased coagulation time for XLC versus NC due to inhibition of the caseinomacropeptide release. When the NC and XLC is consumed in a food matrix, such as cheese spread on a slice of bread, the gastric coagulation was much less expressed.

The digestibility of NC and XLC is expressed as the amount of nitrogen that was dialyzed from the intestinal compartment of TIM as small peptides and amino acids over a membrane with a cutoff of less than 5−7 kDa (bioaccessible amount). Comparing NC and XLC, no significant differences were found in digestibility and bioaccessibility of nitrogen when administered as solubilized protein or in cheese spread on a slice of bread under simulation of the conditions of young children and adults (Figure 2). However, a trend was found for a higher bioaccessibility of nitrogen from XLC versus NC.

Under adult conditions lower amounts of bioaccessible nitrogen were found for both NC and XLC in solution as compared to NC and XLC in the cheese spread. Most likely this was due to the higher gastric coagulation of the solubilized caseinate versus the caseinate in the cheese spread. The clotted proteins are not emptied from the stomach, neither in vivo nor in the TIM system. This is supported by the findings under young child conditions with less coagulation, showing a similar digestibility for NC and XLC in solution and in cheese spread.

The results of the α-amino-nitrogen analysis showed no differences between NC and XLC for alpha-amino nitrogen, for both types of products as well as for both age groups. This indicates that these proteins were digested to similar 'numbers' and sizes of peptides. The NC and XLC in solution, however, showed a higher bioaccessibility for α-amino nitrogen than NC and XLC in the cheese spread. This was found for the absolute concentrations of α-amino nitrogen under young child conditions (with similar concentrations of bioaccessible nitrogen) and for the relative concentration under adult conditions (with lower concentrations of bioaccessible nitrogen). This difference can be related to matrix effects or by the proteins from the wheat bread (22−26% of total protein intake).

The results of these digestibility experiments, showing food matrix differences, support the opinion of Schulten et al.[11] that "the food matrix should be considered when" digestibility and "allergenicity of food allergens is determined".

The breakdown of NC and XLC during pepsin digestion in the stomach of the TIM system was followed by SDS-page gel-electrophoresis. The amount of sample on the gels was corrected for the concentration of solubilized protein (15 μg/lane). The results for NC show clear bands for serum albumin (67 kDa), α-s2, α-s1-, β-, and κ-casein (approx. 32, 30, 29, and 25 kDa, respectively), and the whey proteins ß-lactoglobulin (18 kDa) and α-lactalbumin (14 kDa).[40,41] For XLC it shows the same bands, but less intensive, and an additional band above 200 kDa for the Tgase cross-linked casein.[42] During gastric digestion, the cow's milk proteins bands in NC and in XLC were still visible 90 min after intake (standardized on amount of protein). This was the case under adult conditions as well as under young child conditions for NC and XLC given as solution and in the cheese spread. This demonstrates the resistance of cow's milk proteins against pepsin and the deceasing gastric pH for both products, irrespective of the food matrix and the age groups. These results support the findings of Chatterton et al.[43] showing bovine milk proteins resisting digestion in neonatal gastric juice at pH 4. During digestion of NC new bands are visible after 60 min of gastric digestion, indicating stable breakdown peptides from casein. Also Mandalari et al.[44] reported the appearance of new fragments between 2.5 and 21 kDa during digestion of β-casein by physiological levels of pepsin. Some of these fragments persisted 60 min of pepsin digestion. For NC and XLC given as solution under adult conditions the lanes of the samples taken at 30 and 60 min (lanes 3 and 4) did not show distinct protein bands (Figure 6), most likely related to the coagulation of NC and XLC after 30−60 min at low gastric pH as described above.

The amount of native cow's milk proteins in the XLC products was lower than in the NC products. The smaller amount of native casein in XLC solution was broken down approximately 75−90 min after intake, especially under young child conditions.

The Tgase cross-linked caseinate in the XLC products showed a gradual breakdown in the stomach after 60−75 min. No distinct intermediate peptide bands were found, but more a "smear" of a heterogeneous molecular weight proteins, which was also reported by Monogioudi et al.[9] The glutamyl-lysine cross-link was not broken down to the formation of casein. The cross-link is stable in the GI tract and almost exclusively found in the molecular weight protein fraction of above 200−500 Da.[45] If it is absorbed from the intestine, it can be cleaved by gamma-glutamyl-transpeptidase as present a. o. in the kidney.[46]

The nondigested proteins (if not coagulated) will be gradually emptied from the stomach into the intestinal compartment. The proteins will be digested by the various proteinases (mainly trypsin, chymotrypsin, and elastase) from the pancreatic juice. The results of the gel-electrophoresis of the intestinal samples in time did not show casein bands. Only very distinct and reproducible bands of the secreted proteins are visible. It is expected that the cow's milk proteins are very rapidly digested immediately after emptying from the stomach. This was confirmed by ELISA analysis of selected intestinal samples. As published by Mandalari et al.[44] β-casein was immediately digested by a crude mixture of pancreatin and β-casein fragments were completely digested within 5 min by trypsin and chymotrypsin.

It can be concluded that there was no relevant difference in the total and rate of digestion of NC and XLC in the upper GI tract, measured as the availability for intestinal absorption of total and α-amino nitrogen, neither under simulation of the gastrointestinal conditions of adults nor that of young children. In the gel electrophoresis caseinate proteins could be demonstrated in the lumen samples of the stomach, even 60−105 min after intake. This was found for NC as well as for Tgase XLC containing products. Under young child conditions there was a trend for lower concentrations of casein after 60 min of gastric passage for XLC compared to NC. Under adult conditions no major differences were detected.

In the small intestinal compartment, the caseinate was digested rapidly into small peptides. Neither in the electrophoresis nor in the ELISA, protein bands larger than ∼4 kDa or immune reactive proteins could be detected in the lumen samples collected from the small intestinal compartment. This was found for NC as well as for XLC and for both age groups.

The overall results of this in vitro study demonstrate that the rate of breakdown of the proteins in Tgase XLC and NC food products is not relevantly different.

It is indicated by Astwood et al.[47] and stated by FAO/WHO[48] and Codex Alimentarius[49] that there might be a link between (in vitro) digestion resistance of proteins and potential allergenicity. However, numerous subsequent studies, as reviewed by the EFSA Panel on GMO,[50] have demonstrated that this correlation is not absolute. Allergenicity does not only depend on the digestion of the protein, but also upon the uptake from the gut.[51] This illustrates the need to consider the stability of protein fragments formed under conditions of digestion besides the stability of the intact protein. Of interest are peptides by which the size would be sufficient to allow containing at least two epitopes for binding by multiple IgE-antibodies. Their cross-linking to receptors on the surface of mast cells can lead to release of histamine and other elicitors of allergic reactions. Another possibility is that peptides that are too small to be allergenic by themselves may form aggregates of sufficient size to trigger an allergic reaction.[52] This may play a role in the residual allergenicity of some extensively hydrolyzed infant formulas.[53] So the digestion data generated in this study do not necessarily indicate a lower or even similar potential allergenicity for XLC in comparison to NC products. Also the lack of casein-specific rabbit IgE binding epitopes in XLC and NC digests does not further elucidate the possible differences in allergenicity. Especially the pepsin digested XLC fragments with the stable link between glutamine and lysine should be investigated further.

In conclusion, the kinetics of protein digestion in Tgase XLC food products in Tiny-TIM is not altered as compared to NC food products. The effect of cross-linking of caseinate on the food allergenicity needs to be tested in an animal model for food allergy and demonstrated in allergic individuals.

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Notes

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