



The heat treatment and the gelation are strong determinants of the kinetics of milk proteins digestion and of the peripheral availability of amino acids

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ABSTRACT

This study aimed to determine the kinetics of milk protein digestion and amino acid absorption after ingestion of four dairy matrices by six minipigs: unheated or heated skim milk and corresponding rennet gels. Digestive contents and plasma samples were collected over a 7 h-period after meal ingestion. Gelation of milk slowed down the outflow of the meal from the stomach and the subsequent absorption of amino acids, and decreased their bioavailability in peripheral blood. The gelled rennet matrices also led to low levels of milk proteins at the duodenum. Caseins and β -lactoglobulin, respectively, were sensitive and resistant to hydrolysis in the stomach with the unheated matrices, but showed similar digestion with the heated matrices, with a heat-induced susceptibility to hydrolysis for β -lactoglobulin. These results suggest a significant influence of the meal microstructure (resulting from heat treatment) and macrostructure (resulting from gelation process) on the different steps of milk proteins digestion.

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1. Introduction

The nutritional evaluation of a protein source is based on its composition of indispensable amino acids, and its digestibility over the whole digestive tract (Schaafsma, 2000). However, it is now well established that the speed of the digestion is an important dimension in the definition of this nutritional value. Indeed, quickly and slowly-digested proteins differently affect the metabolic use of dietary amino acids for protein metabolism (Breen & Phillips, 2011; Dangin et al., 2003). The concept of 'slow' and 'fast' proteins mainly derives from comparisons between purified caseins (slow proteins) and whey proteins (fast proteins) (Boirie et al., 1997): caseins are able to coagulate in the acidic environment of the stomach and β -lactoglobulin remains in the serum phase of the bolus. Caseins are therefore thought to be retained

much longer than whey proteins in the gastric compartment, providing a prolonged plateau of moderate hyperaminoacidemia associated to higher protein deposition than whey proteins in young adults (Lacroix et al., 2006). Conversely, in the elderly people, "fast" proteins, such as whey proteins, are more efficient than casein to promote postprandial protein anabolism (Dangin et al., 2003). However, the intrinsic composition of a protein is not the only determinant of its speed of digestion. The structure of the food, also called "food matrix" and defined as the interactions between food components resulting from technological processes during food manufacturing, also impact on this parameter and the subsequent nutrient bioavailability (Parada & Aguilera, 2007; Turgeon & Rioux, 2011).

In this context, the present study aimed to evidence the possibility of modulating the speed of the digestion of dairy products, without changing their biochemical composition, by applying different technological processes acting on the structure of the meal. Two technological treatments commonly used in the food industry were chosen, a thermal treatment and a gelation process, in order to modify both the micro and the macrostructure of the tested matrices. The effects of these structure modifications on the gastric mean retention time, the residual concentrations of milk proteins

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(specifically caseins and β -lactoglobulin) in the small intestine and the postprandial kinetics of aminoacidemia, were investigated in minipigs. This animal model was chosen because its digestive physiology is close to the human one, at least in the upper part of the gut (Rowan, Moughan, Wilson, Maher, & Tasman-Jones, 1994). The results illustrate the strong effects of the food matrix on the gastric emptying, the hydrolysis of the proteins and the kinetics of amino acid absorption.

2. Materials and methods

2.1. General

All procedures were in accordance with the guidelines formulated by the European Community for the use of experimental animals (L358-86/609/EEC, Council Directive, 1986) and the study was approved by the Local Committee for Ethics in Animal Experimentation (Approval CE15-10; CREEA d'Auvergne, Aubière, France).

2.2. Animals and animal housing

The study involved six 18-months-old female adult mini-pigs (20 ± 1 kg). Three weeks before the experimentation, minipigs were surgically fitted with cannulas as described previously by Bauchart et al. (2007) and with a catheter in the abdominal aorta (7 cm inside the blood vessel). They were housed in subject pens (1×1.5 m) in a ventilated room with controlled temperature (21°C). Between the sampling days, they were fed once daily with 400 g/d of a concentrate feed containing 16% protein, 1% fat, 4% cellulose and 5% ash (Porcyprima; Sanders Centre Auvergne, Aigueperse, France) and had free access to water.

2.3. Test meals

Four dairy matrices having a similar composition but differing by their internal structures were prepared from 145 g of Ultra Low Heat (ULH) skim milk powder (4% of humidity, 34% of protein ($N \times 6.25$), 54% of lactose, 8% of ash) manufactured at INRA-STLO (Rennes), rehydrated to 1 kg with Milli-Q pure water. A concentration of about 50 g proteins/kg was chosen for the matrices preparation in order to satisfy animal daily protein requirements with 1 kg (2.5 g proteins/kg BW) and to produce dairy gels with sufficient firmness under coagulation conditions (24 h at 20°C). The coagulation was performed during 24 h because of experimental constraints: meal ingestion occurring at 8:00 a.m., the gelled matrices were prepared the day before the sampling day. The coagulation was also performed at room temperature, in a room located near the housing of mini-pigs. The rehydrated solution was used as liquid matrices, either unheated (LR) or heated 10 min at 90°C (LH). The gel matrices were then prepared from LR (rennet DSM Maxiren 180: 0.003 v/w%) (GR) or LH (rennet DSM Maxiren 180: 0.3 v/w%) (GH). The rennet gelation was performed at 20°C and launched 24 h before the beginning of the *in vivo* experiment. The four matrices were added with chromium-EDTA, a non-hydrolysable and non-absorbable marker of the liquid phase (110.8 ppm, w/w).

2.4. Experimental procedures

The experimental protocol included two periods: the first one for effluent sampling, the second one for blood sampling. In each one, the four dairy matrices were randomly tested on each minipig. Within a period (2 wk), the days of sampling were separated by at least 2 d. Test meals (1 kg of freshly prepared matrix) were offered

for 10 min, and were always consumed entirely within that time. Minipigs had no access to water from 1 h before to 7 h after the meal delivery. Duodenum and jejunum effluent samplings were performed on the same sampling day. Effluents were collected in plastic bottles 30 min before and 0, 20, 50 min, 1 h 45, 2 h 45, 3 h 45, 5 h 15 and 6 h 45min after the beginning of meal ingestion for the duodenum and 15 min before and 15, 35 min, 1 h 05, 1 h 30, 2 h 30, 3 h 30, 5 h and 6 h 30min after the beginning of meal ingestion for the jejunum. The sampling was stopped when 40 ml were collected or after a maximum of 10 min sampling. Collected effluents were weighed, added with a protease inhibitor (0.37 mg phenylmethanesulfonyl fluoride/ml) and homogenised with a stirrer. A first fraction of the homogenate (about 5 g) was used for dry matter determination (24 h at 100°C). A second fraction (about 20 g) was directly frozen in liquid nitrogen and used for SDS-PAGE and ELISA analyses. The remaining of the homogenate was freeze-dried and used for chromium analysis. Blood sampling was performed 60 min, 30 min before and 15, 30 min, 1, 2, 3, 4 h, 5 h 30min, 7 h after the beginning of meal ingestion. Blood samples (2.7 ml) were collected in tubes containing lithium-heparin (S-monovettes, Sarstedt, Marnay, France) and immediately centrifuged at 3000g for 10 min at 4°C . Supernatant was frozen in liquid nitrogen and stored at -80°C .

2.5. Sample analysis

2.5.1. Chemical analyses

The total nitrogen (total N), non-protein nitrogen (NPN) and non-casein nitrogen (NCN) of the matrices before ingestion were analyzed according to the Kjeldahl method (FIL-IDF international standard 20B:1993 and 135A:1998 and FIL-20-1 ISO8968-1 for total N; FIL-IDF international standard 29:1964 and ISO 17997-1 IDF 29-1 for NCN; FIL-IDF international standard 20B:1993 and ISO 8968-4 FIL20-4 for NPN). Freeze-dried duodenal effluents were used for chromium analysis. After homogenisation, 1 g of effluent was ashed at 550°C for 12 h. Chromium was extracted from ashes using 10 ml of an acidic mixture (HCl 1.5 mol/l, HNO_3 1.5 mol/l). The mixture was filtered, added with 5 ml of KCl 0.5 mol/l, and diluted to 50 ml with distilled water. The chromium content was then determined using an atomic absorption spectrometer (AAnalyst 400 Spectrometer, PerkinElmer, Courtaboeuf, France). For amino acid analyses, after thawing, 500 μl of plasma were deproteinised with dried sulphosalicylic acid (55 mg/ml plasma), after the addition of norleucine (50 μl , 1.25 mmol/l), used as an internal standard. Samples were mixed and allowed to stand 15 min at room temperature. Precipitated proteins were removed by centrifugation (10,000g for 10 min at 4°C). The supernatant was then diluted (2/3) with the lithium injection buffer (Bioritech, Guyancourt, France), containing glucosaminic acid, used as an injection standard. Plasma amino acid concentrations were determined with an amino acid analyzer (Bio-Tek Instruments A.R.L., St. Quentin Yvelines, France) by ion-exchange chromatography, with post-column derivatisation with ninhydrine (Slocum & Cummings, 1991). For LR and GR matrices, cholecystokinin (CCK) and ghrelin concentrations in plasma were measured with ELISA kits (BlueGene Biotech CO., Shanghai).

2.5.2. Rheological measurement of the formation of rennet milk gels

Formation of the rennet-induced gels was monitored by measuring the elastic modulus (G'), the viscous modulus (G'') and the loss tangent ($\tan \delta$, defined as the ratio of the viscous to the elastic modulus of a system, G''/G') according to the procedure previously described by Morand, Guyomarç'h, Pézenne, and Famelart (2011) at 20°C with a strain of 0.1%. The onset of gelation was defined as the time when $G' > 1$ Pa. After 24 h of gelation, the effect of the time scale of the applied strain on the rheological properties of

the set gel was determined by varying the frequency from 0.01 to 10 Hz. All experiments were performed at least in duplicate.

2.5.3. Compression test of the final rennet milk gels

Immediately after rennet dispersion, 6 × 25 ml aliquots of the suspension were collected and gelation was carried out during 24 h at 20 °C. After 24 h, compression tests were performed at 20 °C with the 4501 INSTRON universal testing machine using the series IX software (Instron SA, Guyancourt, France) fitted with a 10 N sensor and a 2 cm-diameter stainless steel ball geometry. The procedure involved a penetration step of 20 mm into the sample at a speed of 120 mm/min. The force (mN) and depth of the ball in the gel (mm) at the breaking point were then recorded.

2.5.4. Image analysis of the rennet milk gels microstructure

The unheated or heated milk was added with a rhodamine-B-isothiocyanate (RITC) solution prepared at 85 g/l DMSO: 25 µl of the RITC solution was added to 10 ml of milk and the system was stirred continuously for 15 min. Rennet was then added and immediately after dispersion, one drop of the RITC-labelled milk system was loaded on a cassette, covered by a thin layer of paraffin oil to prevent evaporation and incubated at 20 °C for 24 h. After this period, the cassette was observed by confocal microscopy according to the procedure described previously by Morand, Guyomarc'h, Legland, and Famelart (2012) with digital images being recorded at 8 µm depth into the sample. Grey level granulometric methods from mathematical morphology were applied as described previously by Devaux, Bouchet, Legland, Guillon, and Laye (2008) and explained by Morand et al. (2012).

2.5.5. SDS-PAGE

SDS-PAGE was performed on the effluent samples using 4–12% polyacrylamide NuPAGE Novex Bis-Tris 15-well precast gels (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and as described previously by Bouzerzour et al. (2012), without sample centrifugation. Mark 12 Unstained Standard, Invitrogen was used as a molecular weight (MW) marker. The bands were also compared to a mixture of purified proteins prepared at 6 mg/ml (1 mg/ml of each casein αs1, αs2, β and κ, 1 mg/ml of β-lactoglobulin and 1 mg/ml of α-lactalbumin) and loaded at two dilutions 1:4 and 1:10 (referred as proteins mix 1/4 and 1/10 on the gels) and to a sample of the ULH skim milk powder rehydrated at 50 mg proteins/ml and loaded at the dilution 1:64 (referred as M1 1/64 on the gels). Image analysis of SDS-PAGE gels was carried out using Image scanner II (Amersham Biosciences).

2.5.6. Inhibition ELISA

Inhibition ELISA was performed on duodenal and jejunal samples as previously described (Dupont et al., 2010) using caseins and β-lactoglobulin-specific polyclonal antibodies to determine the residual immunoreactivity of each protein during the digestion process. Effluent samples were first ground with a thurax (Ultra Thurax T8 IKA, Fischer Scientific, 20,000 tr/min–5 min). Duodenal samples analyses were performed in triplicate and jejunal samples were analyzed only once, due to the low concentration of both proteins in these samples.

2.6. Calculations and statistical analyses

The mean retention time (MRT) in the stomach was calculated by a non-compartmental method. The calculation of the MRT with this method does not require a specific hypothesis concerning the marker excretion pattern and is often referred to as the method of moments. The equation of Thielemans, François, Bodart, and Thewis (1978) was used for duodenal samples, where Cr_i is the chromium concentration at time t_i after ingestion of the marked meal:

$$MRT = \frac{\sum_{i=1}^N t_i Cr_i}{\sum_{i=1}^N Cr_i}$$

Time and time × matrix effects on arterial concentrations of amino acids and gastrointestinal hormones were tested using the repeated option of the PROC MIXED procedure of SAS (SAS/STAT Users Guide®, Release 8.1; SAS Institute Inc., Cary, NC, 2000), with minipigs as random effect and time, matrix, time × matrix as factors. When significant time × matrix interaction was found, the LSMEANS procedure was used to test differences at specific times, between groups and within-groups vs. baseline (the mean of the two post-absorptive samples 1 h 30 min before the meal).

Postprandial curve was characterised by its zenith (Y_{max}), by the time at which Y_{max} was observed ($t_{Y_{max}}$), by baseline value (Y_{PA}), and average postprandial concentration (Y_{PP}) calculated from the area under the curve (trapezoidal method). Data were analyzed by ANOVA using the GLM procedure of SAS, with temperature (unheated and heated) and matrix (liquid or gel), and their interaction as factors. Data are presented as means ± SEM (standard error of mean). Differences were considered significant for $P < 0.05$.

The statistical analyses (PCA and FDA) were performed with R 2.12.1 on chromium, caseins and β-lactoglobulin concentrations at the duodenum and on amino acids concentrations in the plasma. The protein concentrations were normalised in order to limit analytical variability. A 24 lines × 9 columns table was created for each data set with the lines as subjects × dairy matrices and the columns as the sampling times. After performing the PCA, an unsupervised clustering method (kmeans) was employed while keeping the first five components of the PCA. Wilks test was used to confirm the relevance of FDA and Fisher test to determine the sampling times having the highest influence on the kinetic differences.

3. Results

3.1. Nitrogen content of the matrices

The heated matrices showed a decrease in NCN values compared to unheated matrices (Table 1), this effect being explained by the interactions of whey proteins with casein micelles during the heat treatment. Therefore, the level of heat-induced whey protein denaturation reached 55% for LH.

3.2. Viscoelastic parameters of the gelled matrices and compression results

The final G' of GH and GR was quite similar (Supplementary data Fig. 1) and frequency sweep measurements performed on these gels were characteristic of weak gel profiles (Supplementary data Fig. 2). The compression test was a complementary analysis at large deformation, which showed the resistance of the gels against the penetration of a stainless steel ball, described by the force and the depth of the ball in the gel at the breaking point. GR was slightly more resistant with a force of 977 mN (vs. 627 mN for GH) observed at a breaking point located at a depth of 10.2 mm (vs. 9.6 mm for GH). From these results, both gels displayed approximately the same properties of viscoelasticity and texture.

3.3. Image analysis of the rennet gels microstructure

GH and GR were visualised after 24 h of gelation by confocal microscopy (Fig. 1). Caseins coagulated in a network (white) surrounding lactosera areas (black) containing minerals, whey proteins and lactose. It seems that both gels had a similar microstructure, with GR having a slightly looser network than

Table 1
Total nitrogen, non-protein nitrogen (NPN), non-casein nitrogen (NCN), protein content and level of whey protein denaturation for liquid and gel matrices from unheated or heated milk product.

Matrices ^a	Total N (g/kg)	NPN (g/kg)	NCN (g/kg)	Proteins ^b (g/kg)	Caseins ^c (g/kg) (%)	Whey proteins ^d (g/kg) (%)	Denatured whey proteins (%)
LR	8.42	0.43	1.92	49.9	37.9 (76)	12.0 (24)	–
LH	8.39	0.43	0.83	49.8	44.5 (89)	5.3 (11)	55
GR	8.48	0.58	2.23	49.4	35.4 (72)	14.0 (28)	–
GH	8.75	0.50	1.41	51.6	42.8 (82)	8.8 (17)	–

^a LR and LH are unheated and heated liquid matrices, respectively; GR and GH are gel matrices from unheated and heated liquid matrices, respectively.

^b Proteins = (total N – NPN) × 6.25.

^c Caseins = proteins – (NCN × 6.25).

^d Whey proteins = proteins – caseins.

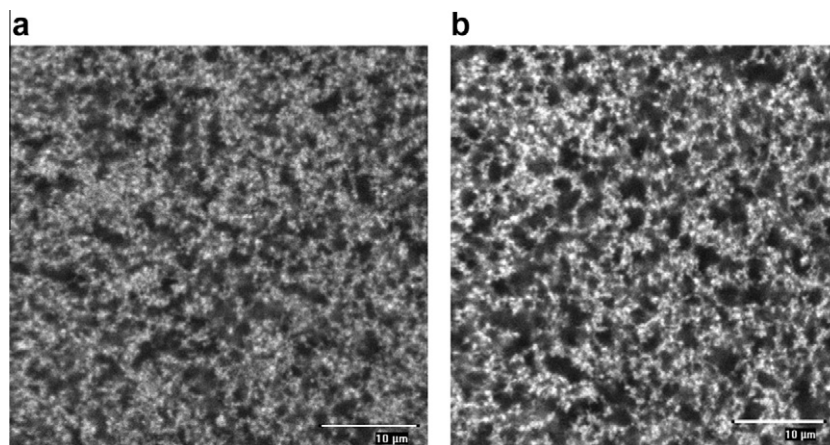


Fig. 1. Confocal scanning laser micrographs of GH (a) and GR (b) milk product, after 24 h of gelation. Bar scale = 10 µm.

Table 2
Descriptive parameters of the curves against time of chromium concentration in duodenal digesta, and indispensable amino acids concentration in arterial plasma, after ingestion of liquid and gel matrices, from unheated or heated milk product, in minipigs.^a

	Liquid (L)		Gel (G)		P		
	Unheated (R)	Heated (H)	Unheated (R)	Heated (H)	L vs. G	R vs. H	LG*RH
<i>Digesta^b</i>							
[Cr]max	87.3 ± 4.4	72.5 ± 12.3	56.0 ± 7.0	47.9 ± 8.0	***	NS	NS
t[Cr]max	11.7 ± 4.2	5.0 ± 0.0	18.3 ± 4.2	45.8 ± 15.1	***	NS	**
MRT	72.6 ± 8.0	96.9 ± 14.3	77.5 ± 10.0	126.4 ± 9.0	NS	**	NS
<i>Plasma^c</i>							
[IAA]max	2678 ± 240	3036 ± 316	872 ± 71	1508 ± 153	***	*	NS
t[IAA]max	45 ± 9	45 ± 9	82.5 ± 23	82.5 ± 33	NS	NS	NS
[IAA]PP	976 ± 32	1387 ± 70	399 ± 77	655 ± 66	***	***	NS

^a Values are means ± SEM, *n* = 6 for digesta, *n* = 4 for plasma.

^b [Cr]max = maximal Cr concentration in the duodenum (µg/g); t[Cr]max = time when Cr concentration is maximal (min); MRT = mean retention time of Cr in the stomach (min).

^c [IAA]max = maximal increase in indispensable amino acids (IAA) concentration in plasma (µmol/l); t[IAA]max = time when IAA concentration is maximal (min); [IAA]PP = average increase in plasma IAA over the postprandial period.

NS *P* > 0.05.

* *P* < 0.05.

** *P* < 0.01.

*** *P* < 0.001.

GH: the granulometric method from mathematical morphology gave a pore size of 0.8 µm and 1.4 µm for GH and GR, respectively.

3.4. Chromium outflow from the stomach

Chromium was used as a marker of the liquid phase of the meal. However, its profile was also highly correlated to the dry matter of the duodenal effluents (not shown). The observations of the kinetics of the duodenal appearance of the chromium and the MRT

values underlined two strong effects on the meal transit, the first one being related to the meal macrostructure (as modified by the gelation process) and the second one to the meal microstructure (as modified by the heat treatment). The gelation process, with both unheated or heated milk products, slowed down the outflow of chromium from the stomach, as illustrated by the decrease in [Cr]max, and the increase in t[Cr]max (Table 2). The heat treatment increased the MRT of chromium in the stomach over the whole postprandial period, of both the liquid and gel matrices.

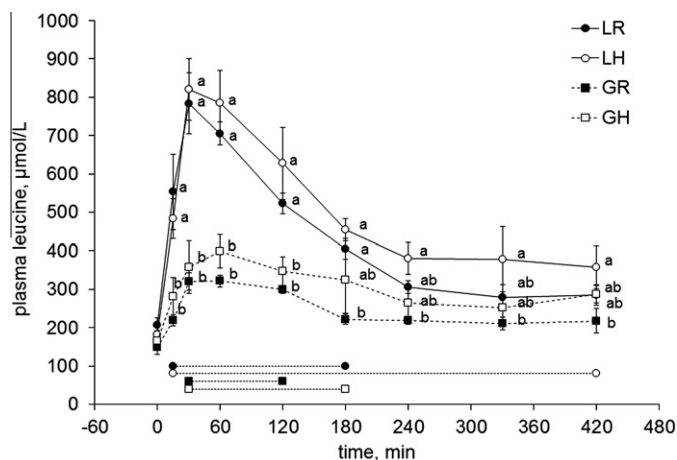


Fig. 2. Plasma leucine concentration ($\mu\text{mol/L}$) over a 7 h-period after ingestion of liquid (L) and gel (G) matrices, from unheated (R) or heated (H) milk product, in minipigs. Values are means $\pm$ SEM calculated for four minipigs ($n = 4$). Data were analyzed by a mixed-model ANOVA. Time effect was significant ($P < 0.001$) and lines at the bottom of the graph indicate a significant difference ($P < 0.05$) from baseline for each curve. Time $\times$ matrix interaction was significant ($P < 0.001$) and at a given time, differences between matrices is evidenced by different letters a and b ($P < 0.05$).

3.5. Plasma amino acids

The presented data focus on the plasma concentrations of indispensable amino acids (IAA: His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val), whose variations, at least during the first hours after the meal, are closely related to their absorption from the gut. Fig. 2 shows the kinetics of absorption of leucine after the ingestion of the four matrices (the absorption profiles of IAA and total amino acids are quite similar, not shown). Two different behaviours were observed, depending on the macrostructure of the matrix ingested (liquid vs. gel). The slope of the curves between 0 and 15 min discriminated the liquid and gelled matrices: the rate of leucine absorption being 22.9 ± 2.9 and 7.5 ± 1.8 $\mu\text{mol/min}$, respectively. Furthermore, the gelation process induced a decrease in the maximal values of IAA observed postprandially in the plasma, and finally a decrease in the average IAA concentration over the postprandial period (Table 2). The effect of heat treatment was less significant, nevertheless it induced an increase in the maximal, and average, IAA concentration in the plasma after the meal.

3.6. Plasma CCK and ghrelin

Kinetics of CCK (anorexigenic) and ghrelin (orexigenic) plasma concentrations are presented in Fig. 3. Although time $\times$ matrix interaction was not significant ($P = 0.17$), it seems that the increase in CCK concentration is faster with LR than with GR and that GR induced a more prolonged increase in CCK than LR. Time $\times$ matrix interaction was also not significant for ghrelin ($P = 0.30$), nevertheless the postprandial decrease in ghrelin concentration was more pronounced with GR than with LR.

3.7. Protein content of effluents determined by inhibition ELISA

ELISA allows the determination of the residual concentration of intact proteins and large protein fragments recognised by the antibody in the effluents. The comparison of the profiles of the residual concentrations of caseins and β -lactoglobulin at the duodenal site after the ingestion of the four matrices is shown by Fig. 4a and b, respectively. LR, LH and GR induced a peak of caseins

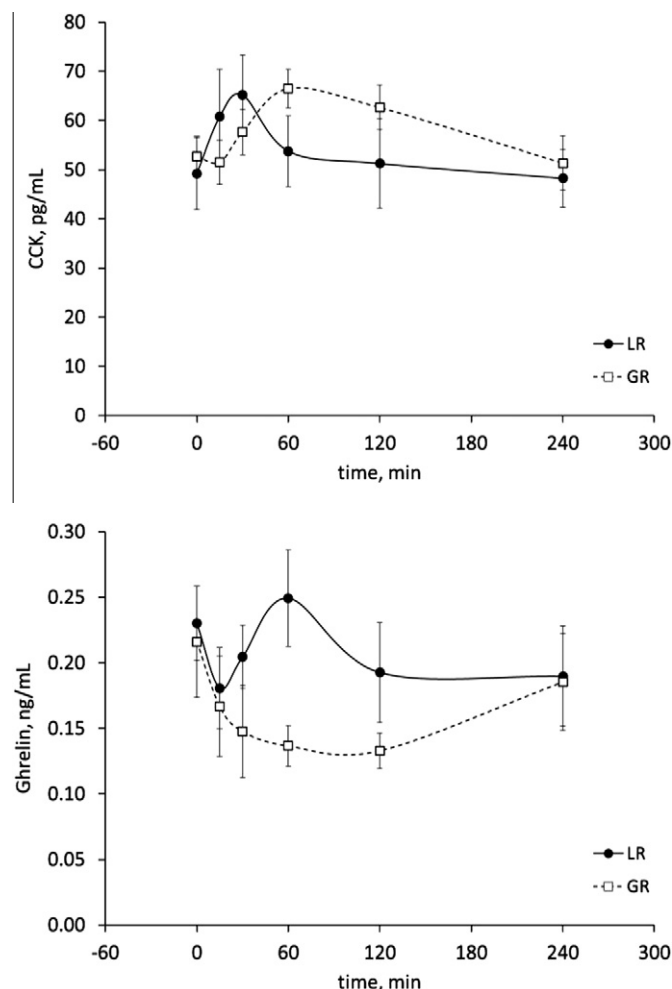


Fig. 3. CCK and ghrelin plasma concentrations over a 7 h-period after ingestion of liquid (L) and gel (G) matrices, from unheated (R) or heated (H) milk product, in minipigs. Values are means $\pm$ SEM ($n = 4$). Data were analyzed by a mixed-model ANOVA. Time effect and time $\times$ matrix interaction was not significant ($P > 0.10$).

in the duodenum shortly after meal ingestion, whereas caseins seemed to be slightly more retained in the stomach after GH consumption (caseins peak at 20 min) (Fig. 4a). LH also induced a late increase of caseins concentration at 3 h 45 min after ingestion. β -Lactoglobulin showed a fast duodenal appearance after feeding LH (Fig. 4b). Strikingly, both proteins concentrations were very low during the whole sampling period after the rennet gels (GR and GH) consumption (Fig. 4a and b). The concentration of residual caseins and β -lactoglobulin at 3 m downstream from the pylorus, in the mid-jejunum, were also very low for all the matrices (≤ 2 mg/ml during the whole digestion), except 15 min after the ingestion of LR, for which a peak is observed at 3.8 and 2.3 mg/ml for caseins and β -lactoglobulin, respectively (not shown).

3.8. Protein content of effluents determined by SDS-PAGE

Fig. 5a and b depicts typical duodenal protein profiles observed by SDS-PAGE after ingestion of LR and LH, respectively. The first sampling (30 min before the ingestion) reflects the endogenous proteins of the bolus. In spite of very saturated bands related to the complexity and the heterogeneity of the duodenal samples, some trends could be observed. After ingestion of LR (Fig. 5a), caseins appeared during the first minutes of digestion (lane

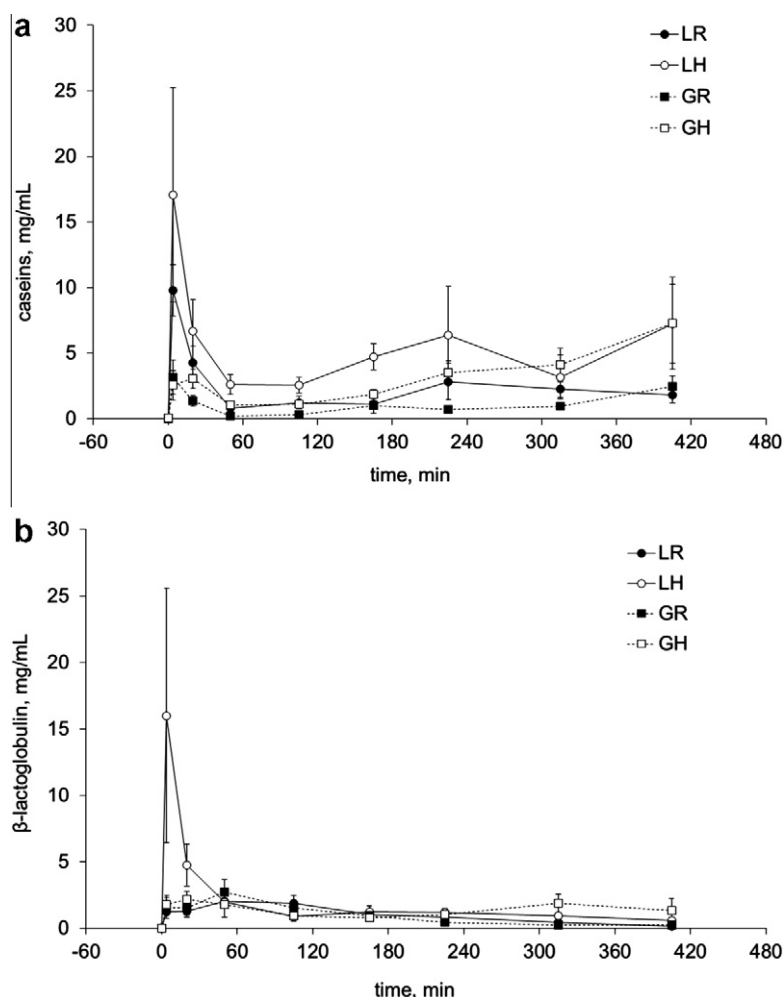


Fig. 4. Caseins (a) and β -lactoglobulin (b) concentrations (mg/ml) of duodenal samples collected over a 7 h-period after ingestion of liquid (L) and gel (G) matrices, from unheated (R) or heated (H) milk product, in minipigs, and determined by ELISA assays (mean $\pm$ SEM based on three independent experiments).

0 min) and were rapidly hydrolyzed during the next 20 min. Conversely, β -lactoglobulin seemed resistant to digestion until 2 h 45 min after the beginning of ingestion. The heat treatment induced a similar behaviour of caseins kinetics to LR, intact caseins being undetectable after 20 min (Fig. 5b). However, later in the digestion process, a smear appeared at 2 h 45 and 3 h 45 min, probably consisting of smaller peptides resulting from caseins proteolysis. Moreover the β -lactoglobulin appeared less resistant to hydrolysis after ingestion of LH, the corresponding band being undetectable after 50 min of digestion (comparing large-dashed lines from Fig. 5a and b). SDS-PAGE profiles after the ingestion of GR and GH gave similar profiles to that of LR and LH, respectively (not shown). Protein profile at the jejunal site suggested that both β -lactoglobulin and caseins were completely hydrolyzed when arriving in this part of the gastrointestinal tract after ingestion of the four matrices (not shown).

3.9. PCA and FDA

From the gastric MRT and the quantification of caseins, β -lactoglobulin and amino acids, it seems that the thermal treatment of processed matrices was a key factor for the protein hydrolysis and that the rennet gelation process had a strong impact on the amino acid absorption and bioavailability in plasma. PCA and FDA were thus performed on these data to highlight potential dif-

ferences between unheated and heated matrices for the protein pool and between liquid and gelled matrices for the amino acid pool. For PCA, the first two principal components represented more than 70% of the variance. Moreover, for each matrix a method of clustering was employed to underline similar or different behaviours between both proteins. For unheated matrices (LR and GR) (Fig. 6a and c, respectively) the dynamics of both proteins were clearly different (as shown by two different clusters): the first axis discriminated the beginning of the kinetics with the concentrations of caseins and β -lactoglobulin reaching their maxima at 4 and 50 min, respectively. In contrast to the unheated matrices, the heated ones (LH and GH) (Fig. 6b and d, respectively) induced similar behaviour of both proteins for each pig. The FDA carried out on leucine data (Fig. 6e), indispensable and total amino acids (not shown) discriminated the behaviours of liquid and gelled matrices. FDA allowed maximizing the variance between groups: FDA is a PCA carried out on the barycenters of *a priori* groups, which are here the four dairy matrices. Wilks test gave a *P*-value of 2.2×10^{-5} for leucine and Fisher test showed that the sampling times until 4 h after meal ingestion allowed discriminating in a significant way two groups (liquid vs. gel) on the first axis. Both groups of matrices were thus distributed along this axis according to the level of leucine measured at the beginning of digestion: the liquid showed higher values than gelled matrices. The FDA and PCA analyses thus confirmed the hypothesis of a significant influence of

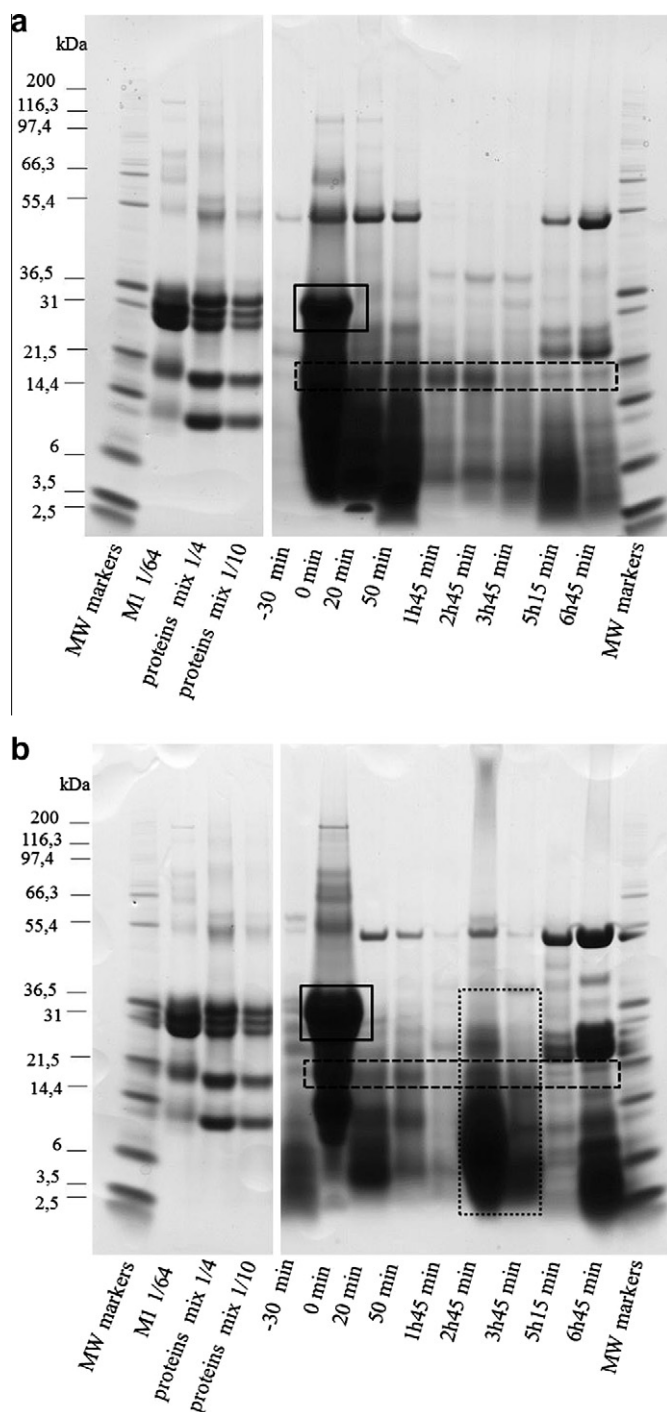


Fig. 5. SDS-PAGE protein profile after ingestion of LR (a) and LH (b) at the duodenum (solid line: caseins, large-dashed line: β -lactoglobulin, small-dashed line: products of casein digestion).

the gelation and thermal treatments of the dairy matrix on the dynamics of proteins transit, hydrolysis and absorption.

4. Discussion

Among food proteins, bovine milk proteins constitute a highly digestible source of dietary nitrogen for humans (ileal true digestibility of milk proteins is estimated to reach 95%, (Gaudichon et al., 2002)) and are precursors of peptides exhibiting bioactive signals

involved in different physiological functions (Korhonen, 2009). The main milk proteins are caseins and whey proteins, of which β -lactoglobulin is the major one (Farrell et al., 2004). Interestingly, β -lactoglobulin is rich in leucine, a branched-chain amino acid able to efficiently stimulate muscle protein synthesis, and thus extensively studied in the context of sarcopenia, a pathological state resulting in a muscle mass loss in the elderly people (Rieu et al., 2006).

This study aimed at understanding the influence of the structure of dairy matrices on the digestion kinetics of the main milk proteins, caseins and β -lactoglobulin. The matrix structure can be observed at the molecular level related to the microstructure (the way proteins interact together) and at the macroscopic level related to the macrostructure (liquid vs. gelled matrices). The potential impact of two technological processes commonly used in the dairy industry, the heat treatment at 90 °C during 10 min and the rennet gelation process, on the different steps of the digestion (gastro-duodenal transit, hydrolysis and absorption) was thus investigated.

This study showed that the heat treatment of a milk product increases its mean retention time in the stomach, and that the gelation process allows delaying in time its gastric outflow. Finally, the gelled matrix from the heated milk product was the most stomach-retained matrix (129 min).

The analysis of the residual concentrations of both proteins was performed by using SDS-PAGE and inhibition ELISA. A pragmatic approach was used to draw information from this complex dataset with data normalisation and statistical analyses. The comparison of the unheated and heated matrices led to the conclusion of a significant influence of the heat treatment on the hydrolysis of both caseins and β -lactoglobulin. After ingestion of the unheated matrices (LR and GR), each protein had its own behaviour with β -lactoglobulin and caseins being respectively resistant and sensitive to hydrolysis in the stomach. After feeding the heated matrices, both proteins showed similar digestion with a heat-induced susceptibility to hydrolysis for β -lactoglobulin. These results are in accordance with previous *in vitro* studies which showed that caseins are rapidly cleaved by pepsin and pancreatic enzymes (trypsin and chymotrypsin), whereas β -lactoglobulin is highly resistant to proteolysis (Inglingstad et al., 2010; Mandalari et al., 2009) and that a thermal treatment is able to render β -lactoglobulin susceptible to hydrolysis (Kim et al., 2007). Indeed, disruption of native β -lactoglobulin conformation after thermal treatment at 90 °C during 10 min exposes susceptible cleavage bonds (Reddy, Kella, & Kinsella, 1988). The similar digestion observed for both proteins after feeding the heated matrices could be related to the formation of soluble and micelle-bound protein aggregates between β -lactoglobulin and κ -caseins located at the surface of casein micelles, resulting from covalent binding and hydrophobic interactions after thermal treatment (Guyomarc'h, Law, & Dalgleish, 2003). It has recently been proved that protein aggregation could elicit sensitization of allergic patients to milk proteins through Peyer's patches (Roth-Walter et al., 2008): ingestion of heated dairy matrices has thus to be taken into account in the context of allergy to milk. Another effect ascribed to the heat treatment was the potential formation of a firmer coagulum in the stomach, leading to the subsequent emptying of two populations of caseins, one being released in an intact form in the first minutes of digestion process and the other one being emptied later (3–4 h after the meal ingestion) in the form of peptides resulting from the caseins peptidolysis in the stomach. The second population of caseins or caseins fragments could have been stuck against the stomach walls and emptied later in the digestion process during strong stomach wall contractions. This effect was also observed for GH, the amount of caseins collected at the end of the duodenum stea-

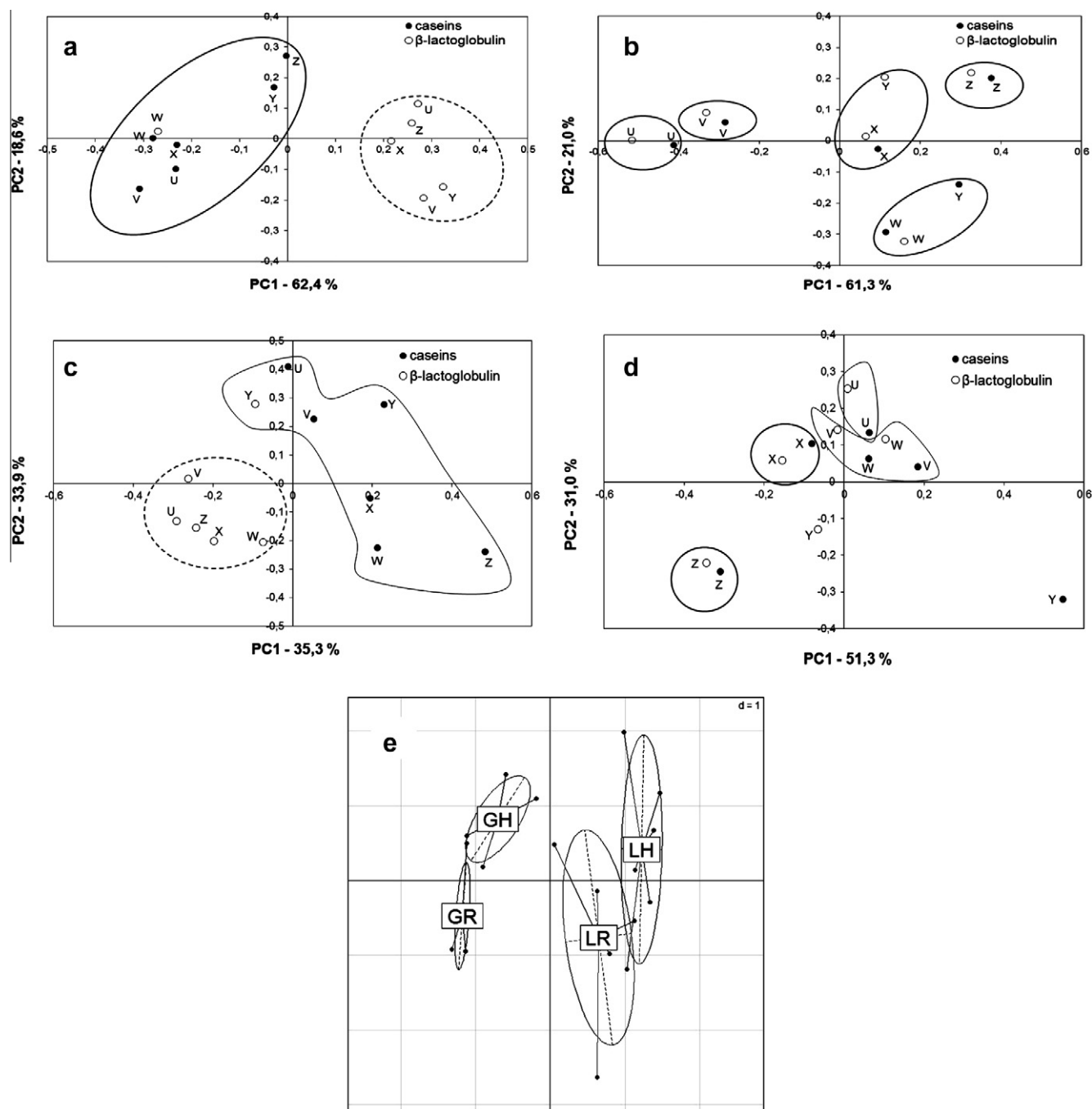


Fig. 6. PCA performed on caseins and β -lactoglobulin data after the ingestion of liquid (L) and gel (G) matrices, from unheated (R) or heated (H) milk product (a: LR; b: LH; c: GR; d: GH); FDA performed on leucine data (e). Each point represents the kinetics of caseins or β -lactoglobulin digestion after the ingestion of a matrix by a pig (named U, V, W, X, Y or Z).

dily increasing from 4 h after meal feeding until the end of the sampling period. Interestingly, β -lactoglobulin did not seem to be split into two populations like caseins, suggesting that the hydrolysis of both proteins associated after the heat treatment occurred by different mechanisms according to the molecular structure of each protein. More studies are thus necessary to understand the fate of caseins- and β -lactoglobulin-originating peptides after both parent proteins have been associated as a result of a thermal treatment.

The endpoint of the digestion process is the absorption of amino acids. The bioavailability of amino acids after meal ingestion was

evaluated by plasma indispensable amino acids concentrations, and more particularly leucine. The meal macrostructure (i.e. liquid vs. gelled matrices) had a higher influence on the rate of amino acid appearance in the blood than the meal microstructure (i.e. unheated vs. heated matrices). The gelled matrices induced delayed and lower levels of amino acid absorption than liquid matrices throughout the plasma collection period (i.e. 7 h after the meal ingestion). This delayed and lower amino acid bioavailability after gelled matrices ingestion could have substantial effect on the protein metabolism at the splanchnic and peripheral levels. Indeed, plasma aminoacidemia is known to stimulate the muscle protein

synthesis, and it was shown that the threshold for the stimulation of muscle protein synthesis is increased in elderly people, and not often reached with an average protein diet in elderly (Breen & Phillips, 2011). This could contribute to the establishment of sarcopenia, a progressive loss of muscle mass in elderly people. Nutritional strategies were thus developed to increase the postprandial aminoacidemia in order to restore the stimulation of protein synthesis (Mosoni & Patureau Mirand, 2003) and it was shown in the elderly that rapidly digested proteins are more efficient to stimulate postprandial protein synthesis than slowly digested proteins (Dangin et al., 2003). The ranking of dietary protein according to digestion rate is uneasy. Our results show that for similar intrinsic composition, this parameter can be altered according to the nature of the matrix. From the present study, it seems that ingestion of liquid matrices, more than gelled matrices, would be preferable to induce an efficient muscle protein synthesis for this specific population. Furthermore, although we have not enough data in this experiment in order to clearly show it, the nature of the matrix seems to affect the release of the gastro-intestinal hormones involved in appetite regulation, the gel-matrix appearing to be potentially more satiating. In the context of weight-reducing diets, the gel-matrices could thus be more interesting. Caseins and whey proteins differently affect the release of gastrointestinal hormones (Bowen, Noakes, Trennery, & Clifton, 2006; Hall, Millward, Long, & Morgan, 2003; Tessari et al., 2007). The present study showed that the kinetics of this release can also be modulated by applying different processes on a same mix of these milk proteins.

The ingestion of rennet-gelled matrices led to very low levels of both proteins at the duodenal site and of amino acids in the plasma. The formation of a coagulum with a high stiffness, under the simultaneous action of the stomach acidity and the rennet, could lead to a very long retention of the meal in the stomach. This is supported by the MRT value of GH, which is the most retained matrix in the stomach, and by the fact that amino acid concentrations have not yet returned to their post-absorptive values, even after 7 h of digestion. The only other *in vivo* study to our knowledge having investigated the effect of dairy product structure on the nitrogen kinetics showed that both the intestinal delivery of the liquid phase and the nitrogenous fraction of the chyme were delayed more in pigs fed yogurt than in pigs fed milk (Gaudichon et al., 1994). The low levels of both proteins and amino acids after the rennet-gelled matrices ingestion could thus be related to a long residence time in the stomach probably associated to higher gastric secretions over a longer period than the liquid matrices. However, further *in vitro* and *in vivo* studies would be necessary to unravel the mechanisms underlying the low duodenal concentrations of proteins and the low bioavailability of amino acids over time after ingestion of these rennet matrices.

In the context of food nutritional properties, this study underlined the strong impact of the meal micro- and macrostructure, resulting from technological processes used in the food industry, on the different steps of milk protein digestion. The food matrix designing at the technological level is thus of particular interest in the control of nutrients delivery, especially for specific subpopulations, such as the elderly or the overweighted people.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.09.022>.

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Supplementary data

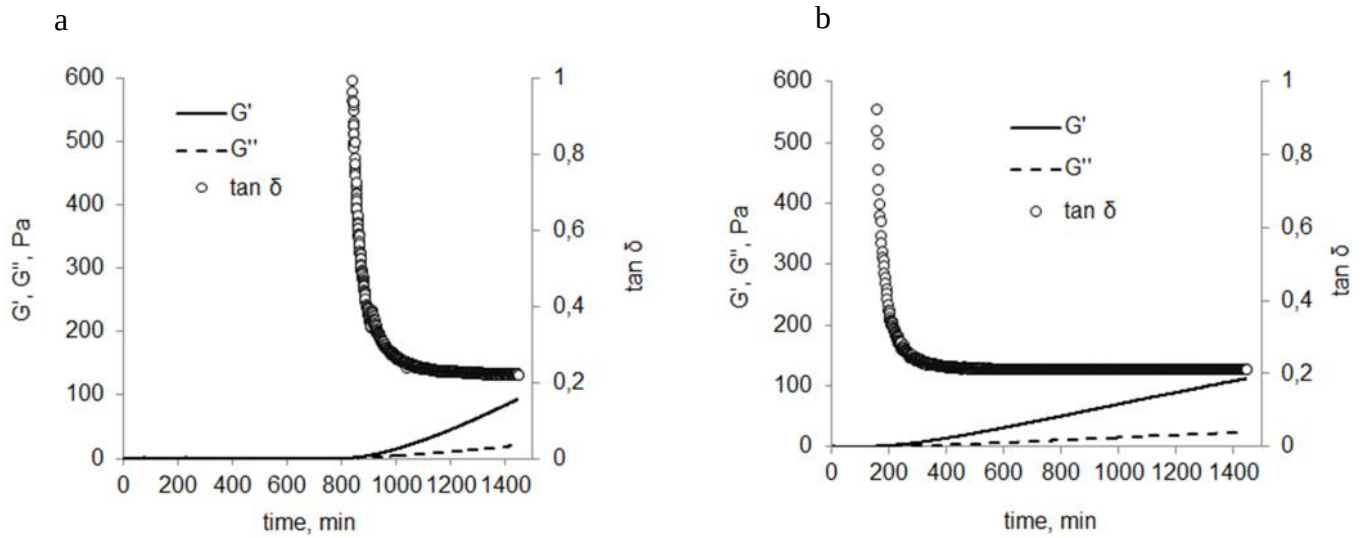


Figure 1: Low amplitude dynamic oscillation measurements over the 24h gelation period of GR (a) and GH (b) (G' : elastic modulus; G'' : viscous modulus; $\tan \delta$: G''/G' , duplicates gave similar profiles).

The final G' of GH and GR was quite similar (112 and 93 Pa for GH and GR, respectively), with a loss tangent δ (G''/G') of about 0.2. The onset of gelation took place earlier for GH than for GR (3 h vs. 14 h) owing to the different concentrations of rennet used to produce gels of similar firmness (this concentration was hundred times higher for GH than for GR). A pH of 6.6 was recorded constant over the whole gelation process for both gels.

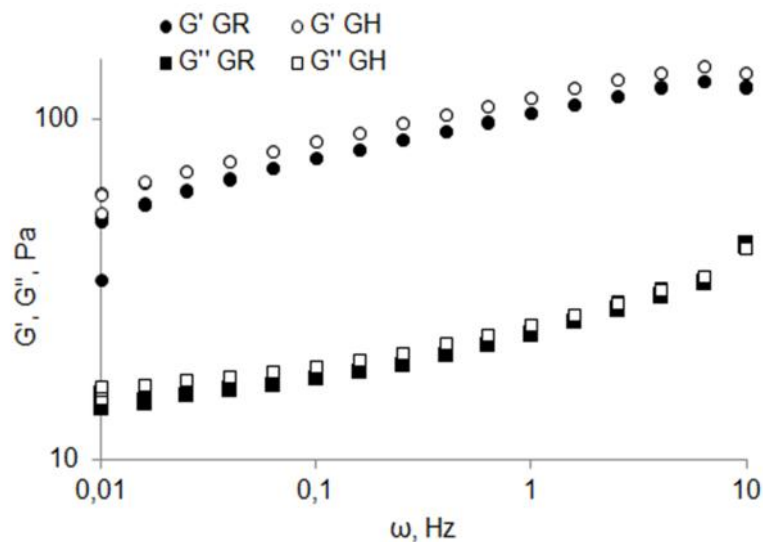


Figure 2 Frequency sweep measurements performed on gel matrices from raw (GR) and heated (GH) liquid matrices (G' : elastic modulus; G'' : viscous modulus, duplicates gave similar profiles; ω : frequency).

The data obtained by frequency sweep measurements performed on GR and GH were characteristic of weak gel profiles, the parameters G' and G'' being slightly dependent on the frequency with a power-law equation having an exponent of ≈ 0.15 , which is in agreement with values reported for other types of weak acid gels (Lucey, Munro and Singh, 1999; Roefs and Van Vliet, 1990).

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