

The Ratio of Casein to Whey Protein Impacts Yogurt Digestion In Vitro

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Abstract Recent findings suggest that the form and texture of food impact appetite and food intake. For example, viscous milk beverages elicit lower food consumption when compared to less viscous beverages. Additionally, individual proteins exert different enzyme susceptibilities that can affect the digestion kinetics of protein and the liberation of satiating peptides and amino acids. The effect of yogurt composition on satiety has been studied, but mostly with regards to added fiber (inulin and guar) and/or increased amounts of protein (Lluch et al. *Food Qual Prefer* 21:402–409, 2010; Perrigue et al. *J Am Diet Assoc* 109:1862–1868, 2009); little information has been reported on the satiating effect of yogurt containing different ratios of casein and whey proteins. Yogurts with different casein to whey protein ratios (4.5:1, 2.8:1 and 1.5:1) were prepared and an in vitro digestion was performed to assess the protein disappearance and amino acid release. A significantly higher viscosity for the 1.5:1 ratio was related to a lower disintegration in the gastric phase. However, all of the ratios were similarly digested at the end of the duodenal phase. After 5 min of duodenal digestion, most of the proteins were completely digested regardless of the ratio. The viscosity at the end of each digestion step was significantly different. Ratio 2.8:1 showed a significantly higher viscosity after oral digestion, while after gastric digestion, both ratio 2.8:1 and 1.5:1 were more viscous than ratio 4.5:1. However, the three yogurt formulations reached a similar viscosity after duodenal

digestion. It can be concluded that the digestion kinetics of nitrogenous compounds are influenced by the viscosity of the food matrix and therefore could impact its satiating properties.

Keywords Yogurt · Casein:whey protein ratio · Rheology · Microstructure · In vitro digestion

Introduction

The rapid increase in the prevalence of overweight and obesity and their associated co-morbidities represent a major global health problem. Healthcare costs are increasing in part due to several obesity-related chronic diseases, such as type 2 diabetes, high blood pressure and cardiovascular diseases. Satiety is one of the approaches used to improve weight management and it refers to the processes that inhibit further eating in the postprandial period. The short-term regulation of food intake results from a series of neural and humoral signals that originate from the gastrointestinal tract in response to the mechanical and chemical properties of ingested foods [11]. Once ingested, a food induces satiety by mechanical stimulation (gastric distension) and by the release of peptides in the intestine [36]. After diffusion through the interstitial fluids, these messengers activate nearby nerve fibers and/or enter the blood circulation to function as hormones [11]. Dairy products, because of their protein content, have the potential to affect satiety by slowing gastric emptying or stimulating the synthesis of gastrointestinal hormones (ghrelin, cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1)) [7, 18, 27, 28]. To exert a nutritional effect in humans, nutrients need to be released from the food matrix (bioaccessibility), transported through the epithelial membrane of the intestine (bioavailability) and metabolized by the liver or other targeted organs [45]. Thus,

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the composition and final structure of a food product is believed to influence the way in which nutrients are digested, released and absorbed and more specifically, the time necessary to render the nutrients available [2, 32, 43].

The structure and composition of food can have an impact on the kinetics of protein hydrolysis and subsequent amino acid absorption. The effect of a food on satiety is dependent on its composition and energy content, but food palatability, physical properties, and structure are also important. The food structure affects the rate and extent of digestion and the rate of the absorption of nutrients. The rate of gastric emptying has been linked to satiety for all forms of food [4]. Recent findings suggest that food form and texture impact appetite and food intake. Solid dairy foods elicit a higher satiety than milk beverages in most studies [19, 21, 25]. The viscosity also affects food intake, where viscous milk beverages elicit lower food consumption [47]. Additionally, individual proteins exert different enzyme susceptibilities. For example, the transition of β -lactoglobulin through the stomach is both fast and resistant to pepsin activity; therefore, the hydrolysis of this protein begins in the upper part of the intestine [5]. Alternately, micellar caseins coagulate in the stomach due to an acidic pH, which allows pepsin to hydrolyze these proteins before their arrival in the intestine. Many studies have looked at the satiating effect of casein and whey protein. Hall et al. [18] reported that whey protein elicits a higher secretion of gut hormones (CCK, GLP-1 and GIP) and greater satiating effects than casein. This finding shows that these two proteins result in different digestion kinetics.

Food processing steps, such as heat treatment, influence the matrix structure and can modify the digestion and absorption kinetics of nutrients. For example, heat treatment could modify the conformation of a protein and consequently its susceptibility to enzymes. Mullally et al. [31] showed that heat-induced unfolding increased the susceptibility of beta-lactoglobulin to proteolysis by pepsin and trypsin. Furthermore, molecular interactions (protein–protein, protein–polysaccharide or protein–lipid) could modify the enzymatic susceptibility of a protein and its behavior in the acidic pH of the stomach. Recently, Lacroix et al. [24] demonstrated that the transformation of dietary nitrogen into serum amino acids, proteins and urea was significantly higher in ultra-high temperature than in the pasteurized milk, suggesting that the digestive kinetics and metabolism of dietary proteins are modified. Thus, the kinetics of protein digestion and the release of amino acids may vary between foods that seem quite similar and different food forms could induce different satiating effects. Viscous (yogurt) and solid (cheese) foods have increased satiety compared to liquids (milk) [25]. The effect of the modification of yogurt composition on satiety has been studied, but mostly with regards to added fiber (inulin, guar) and/or an increased amount of protein [26, 35]. Little information has been reported on the satiating effect of yogurt

containing different ratios of casein and whey protein and the impact of milk heating on the digestion kinetics of yogurt. To assess the effect of milk protein composition on digestion, yogurts of varying compositions were studied. Two heating times were chosen to evaluate the impact of protein denaturation. After the yogurt was manufactured, *in vitro* digestion experiments were performed to assess the bioaccessibility of nitrogenous nutrients. A better understanding of the optimal matrices' characteristics needed to release these key nutrients will support functional dairy foods that could improve weight management in obese individuals.

Materials and Methods

Milk Formulation

Milks with different Casein:whey protein ratios (4.5:1, 2.8:1 and 1.5:1) were prepared using low-heat skim milk powder (Agropur, Canada), whey protein isolate (Davisco, USA), native phospho-caseinate (Ingredia, USA) and alpha-lactose (Alphaeaser, USA). A milk permeate from commercial pasteurized skim milk was prepared using a 10 PM polysulfone UF membrane (5–10 kDa) at 30 °C. These ingredients were used to obtain equivalent amounts percent of protein (3.98 ± 0.02), lactose (9.38 ± 0.03), ash (0.82 ± 0.02), fat (0.06 ± 0.01) and dry matter (14.05 ± 0.05) for each formulation.

The milk formulations were stirred for 2 h and stored overnight at 4 °C. The next day, the pH of the milk formulation was adjusted to 6.6, homogenized (EmulsiFlex C5, Avestin Co., Ontario, Canada) by a single pass at 1,000 psi at 60 °C and heat treated at 90 °C under stirring for 1 or 4 min in a water bath. The milk formulations were cooled to a fermentation temperature (42 °C) before an inoculation with a commercial starter at a concentration of 0.02 % (Yo mix 860, Danisco, Canada). The fermentation lasted for approximately 4 h until the yogurt reached a pH of 4.6. Then, the yogurts were cooled on ice to limit post-acidification and stored at 4 °C for at least 24 h before use. Three independent experiments were conducted for each condition.

Yogurt Analysis

On the day of the experiment, the pH and syneresis of each non-digested yogurt (ND) sample were measured. The pH measurements were conducted with a Symphony pH meter (VWR, On, Canada) and the yogurt syneresis was measured as described previously [22]. Briefly, the yogurt (30 g) was centrifuged at 1,200 rpm for 10 min at 4 °C. The supernatant was weighed and the percent of separated liquid out of the total weight of the sample represents the syneresis.

The protein amount in each yogurt formulation before and after heating was quantified according to standardized

methods [1]. The denaturation of the whey protein was assessed as previously described [39]. It was assumed that the un-denatured whey protein refers to the difference between the soluble protein (SP) measured at a pH of 4.6 and the non-protein nitrogen (NPN) before (i) and after (f) heating. The following calculation was used to determine the percent of whey protein denaturation:

$$\% \text{ whey protein denaturation} = 100 - \left[\left(\frac{SP_f - NPN_f}{SP_i - NPN_i} \right) \times 100 \right]$$

In Vitro Digestion Model

The in vitro oral, gastric and duodenal digestions of yogurt samples containing different casein:whey protein ratios were analyzed, as described previously [45], to mimic human digestion. All of the artificial digestive juices were heated to 37 ± 1 °C prior to use and the digestion temperature was equilibrated at 37 ± 2 °C. Before digestion, 9.00 ± 0.05 g of yogurt was weighed. The digestion was started by adding 6 mL of saliva to the yogurt samples and mixing for 2 min at 75 rpm with a Caframo (Caframo, Canada) equipped with a paddle. Then, 6 mL of gastric juice was added and an orbital rotation (75 rpm) was applied. After 30 min, another 6 mL of gastric juice was added and gastric digestion was allowed to continue for 30 min. Finally, 18 mL of duodenal juice and 2 mL of a bicarbonate solution (1 M) were added for the duodenal phase. The same orbital rotation (75 rpm) was applied for the duodenal phase for 1 h. Control digestions were also realized in the same conditions using water instead of yogurt. The digestion was carried out in beakers that were entirely used for the analyses meaning that one beaker corresponds to one digestion time point. All of the following enzymes were obtained from Sigma (Canada): alpha-amylase porcine pancreas type VI-B (A3176-1MU) at 1.93 units/g of yogurt, pepsin from porcine gastric mucosa (P7000) at 833.3 units/g of yogurt, pancreatin from porcine pancreas (P-7545) at 96 units/g of yogurt, lipase from porcine pancreas (L3126) at 800 units/g of yogurt and porcine bile (B8631) at 0.02 g/g of yogurt.

Samples were collected at intervals to measure the release of proteins and amino acids during in vitro oral (O2), gastric (G5, G15, G30 and G60) and duodenal (D5, D15, D30 and D60) digestion (Fig. 1). At the end of each digestion step, the samples were homogenized with an Ultraturrax T25 (IKA Works, Inc., NC, USA) for 20 s and adjusted to a pH of 7.0. Enzymatic activity was stopped using heat treatment (90 °C for 5 min to inactivate the α -amylase) for O2, or the use of an inhibitor. Samples were cooled on ice and centrifuged at $10,000 \times g$ for 20 min at 4 °C. The supernatants were aliquoted before freezing at -80 °C. From the duodenal supernatants,

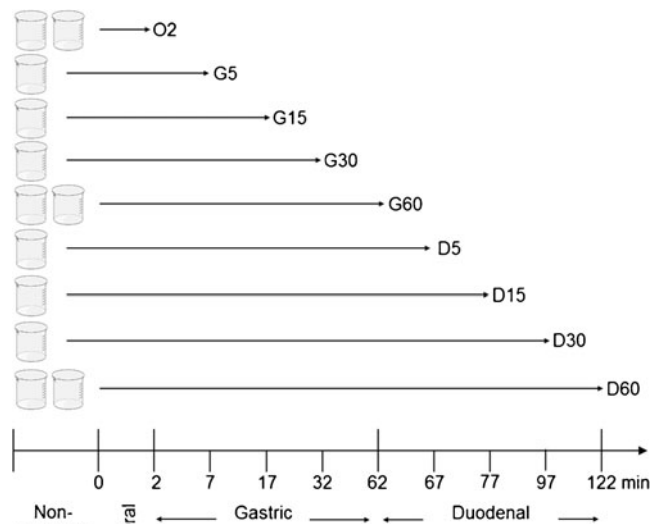


Fig. 1 A schematic representation of the in vitro yogurt digestion model representing the digestion collecting time. A second sample was prepared for the oral (O2), gastric (G60) and duodenal (D60) digestion steps to perform viscosity measurements

4 mL was mixed with a trypsin-chymotrypsin inhibitor (ratio inhibitor/pancreatin of 1:500; T9777, Sigma, Canada) before freezing.

The pellet produced after the centrifugation step was weighed to estimate the yogurt disintegration. A micro BCA test was conducted (according to the supplier recommendations available at <http://www.piercenet.com/instructions/2160412.pdf>) on the supernatants to estimate the SP concentration of the supernatants. The concentration of insoluble protein (IP) in the pellet was also determined by the difference. The amount of protein initially found in the yogurt (Pro_{ini}), the amount of protease added at each digestion step (Pro_{ENZ}), and the amount protein found in the supernatant of the control digestion after O2 or G60 or D5 digestion steps (Pro_{WC}) were used to estimate the insoluble protein concentration.

$$IP = [(Pro_{ini} + Pro_{ENZ}) - SP] - (Pro_{ENZ} - Pro_{WC})$$

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The samples were diluted in a bromophenol blue solution containing β -mercaptoethanol to a final concentration of $2.5 \mu\text{g}/\mu\text{L}$. A total of $20 \mu\text{g}$ of protein were loaded on a 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN Tetra cell system (Bio-Rad, Mississauga, Canada) unless stated differently in the figure caption. An unstained broad molecular weight standard mixture (6.5–200 kDa, Bio-Rad) and a mixture of α -casein, β -casein, κ -casein, β -lactoglobulin

and α -lactalbumin (Sigma, Oakville, Canada) each containing 0.48 μg of protein were loaded on the gel. The gels were stained with Coomassie blue.

Free Amino Acids

The release of amino acids during the in vitro digestion was quantified using an EZ:faast physiological free amino acid analysis kit (Phenomenex, USA) by following the manufacturer's procedures (available at <http://www.phenomenex.com/Products/Detail/EZfaast>). The derivatized samples were analyzed by gas chromatography–flame ionization detector (GC–FID) following the manufacturer's procedures. The derivatives were separated and quantified by GC system (GC-2010, Shimadzu Columbia, MD, USA) equipped with a FID detector and a Zebron ZB-AAA GC (0.25 mm $\times$ 10 m) column in split injection mode using H_2 as carrier gas at a flow of 1.3 mL/min. The oven temperature was programmed at 110 $^{\circ}\text{C}$, ramped at 10 $^{\circ}\text{C}/\text{min}$ to 200 $^{\circ}\text{C}$ and then heated at 32 $^{\circ}\text{C}/\text{min}$ to 320 $^{\circ}\text{C}$ for a total run of 13.75 min. Injector and detector temperatures were maintained at 300 and 320 $^{\circ}\text{C}$, respectively. Quantification was conducted using three amino acid standard mixtures and the calculations were performed by the software controlling the analytical instrument (gas chromatograph). The calculations and calibrations were based on an internal standard.

Rheology Measurements

A controlled strain rheometer (ARES-100 FRT, TA-Instrument, New Castle, DE, USA) equipped with a concentric cylinder (33.93 mm cup diameter, 32.05 mm bob diameter and 33.29 mm bob length) was used to determine the apparent viscosity (Pascal second) before (ND sample) and after each step of yogurt digestion (O2, G60 and D60 samples) using samples taken before the homogenization step. Analyses were performed in a controlled water bath at 37 $^{\circ}\text{C}$. At least three experiments were conducted for each ratio studied. The flow tests were conducted at a rate of 0.1–1,000 s^{-1} . The results are presented as the apparent viscosity (Pascal second) as a function of shear rate (per second). For the non-digested yogurt and oral digestion, the curve modelization was achieved using the power law model, where η represents the apparent viscosity, τ the shear stress and γ the shear rate. The apparent viscosity was linked to the shear stress and shear rate as indicated in Eq. 1:

$$\eta = \frac{\tau}{\gamma} \quad (1)$$

The power law model was expressed by Eq. 2, where k represents the consistency index and n the flow behavior index:

$$\tau = k\gamma^n \quad (2)$$

By substituting Eq. 2 into Eq. 1, the following equation was obtained:

$$\eta = \frac{k\gamma^n}{\gamma} \quad (3)$$

or, upon simplification:

$$\eta = k\gamma^{n-1} \quad (4)$$

$$\log \eta = \log k + (n - 1) \log \gamma \quad (5)$$

where $\log k$ corresponds to the intercept point and $(n-1)$ to the slope of the curve. Moreover, $n < 1$, $n = 1$ and $n > 1$ correspond to a shear thinning, a Newtonian and a shear-thickening material, respectively.

For the gastric samples, the Carreau model was used. This model is expressed by Eq. 6, where η_0 represents the zero shear viscosity, η_{∞} represents the infinite shear viscosity, λ represents the inflection point where the sample behavior changes from Newtonian to shear thinning and n corresponds to the power law exponent describing the shear-thinning behavior of the sample:

$$\tau = \eta_{\infty} \gamma + \frac{(\eta_0 - \eta_{\infty}) \gamma}{\left[1 + (\lambda \gamma)^2\right]^{\frac{(1-n)}{2}}} \quad (6)$$

By substituting Eq. 6 into Eq. 1, the following equation is obtained:

$$\eta = \eta_{\infty} + \frac{\eta_0 - \eta_{\infty}}{\left[1 + (\lambda \gamma)^2\right]^{\frac{(1-n)}{2}}} \quad (7)$$

For the duodenal samples, the apparent viscosity values at shear rates of 31.6 and 100 s^{-1} were used for comparison.

Statistical Analysis

All data are presented as the means $\pm$ SD. A random experimental design was used and analyzed using a measure of the least significance difference using the Statistical Analysis System (version 9.2, SAS Institute, Cary, NC, USA). Differences were considered to be statistically significant at $p < 0.05$.

Table 1 The syneresis and flow curve modelization for yogurt samples with different casein:whey protein ratios and heating times

Physicochemical properties	Heating time		1.5:1	2.8:1	4.5:1
Syneresis (%)	1		0.93±0.99 a	1.29±0.66 a	3.82±1.93 a
	4		1.49±0.94 a	1.91±1.11 a	3.44±0.96 a
Flow curve modelization ^a	1	<i>k</i>	10.2±4.1 a	4.1±2.4 a	2.7±0.2 a
		<i>n</i>	−0.8±0.2 a	−0.4±0.2 a	−0.4±0.0 a
	4	<i>k</i>	11.2±0.7 a	3.3±0.6 b	2.7±0.7 b
		<i>n</i>	−0.9±0.2 a	−0.5±0.1 b	−0.4±0.1

^a The modelization was realized with the power law model and the correlation coefficient was always greater than 0.95

Lines with the same letters are not significantly different at $p < 0.05$

Results and Discussion

Effect of Casein:Whey Protein Ratios and Heat Treatment on Yogurt Physicochemical Properties

The following three casein:whey protein ratios were selected to study the impact of yogurt microstructure on protein digestion: 4.5:1 represents the natural ratio found in milk, 2.8:1 represents the usual ratio for industrial yogurt manufacture and 1.5:1 represent an extreme ratio. After the yogurt was manufactured, the pH was similar for all of the ratios and the heat treatment durations tested, with an average pH value of 4.56 (data not shown). The syneresis of the yogurt samples was not significantly affected by the heat treatment and ratio. However, the higher casein:whey protein ratios showed a tendency toward higher syneresis values (Table 1). This could be explained by the higher water-binding capacity of the denatured whey protein found in higher quantity in higher ratios [30, 37].

The apparent viscosity of each yogurt was also measured. The flow curve for the 1.5:1 ratio showed higher apparent viscosities at low shear rates and the viscosity decreased considerably at higher shear rates (Electronic Supplementary Material (ESM) 1). Ratio 2.8:1 and 4.5:1 showed similar apparent viscosities independent of the heating time used (ESM 1). A curve modelization was then performed using the power law model for each treatment. The consistency index (*k*) and the behavior index (*n*) were compared (Table 1). The effect of the ratio was significantly different for *k* and *n* ($p = 0.0005$ and $p = 0.0017$, respectively), while no significant effect was found for the heating time. Both indices showed that the ratio 1.5:1 was significantly different than the ratios of 2.8:1 and 4.5:1 when a heating time of 4 min was used. A behavior index < 1 is indicative of a shear-thinning material. Thus, the 1.5:1 ratio had a stronger shear thinning behavior than the other ratios. These results suggest that those yogurts possess different microstructures that could impact the availability of the nitrogenous components. Others have reported that the microstructure of yogurt was influenced by the casein:

whey protein ratio, where a high ratio (4.7:1) indicated a network with large pores and contained relatively large size globular aggregates, while the lower ratios indicated a finer network with smaller aggregates and pores [37]. These results were later confirmed by others [16, 39]. It clearly demonstrates that reducing the casein to whey protein ratio modifies the yogurt microstructure and we hypothesized that this could impact protein digestion.

The extent of whey protein denaturation was measured (Table 2). The results showed that the ratios of 2.8:1 (87 %) and 1.5:1 (87–96 %) had a significantly higher denatured whey protein proportion compared to the ratio 4.5:1 (75 %; $p = 0.0008$). The ratio 4.5:1 initially contained less undenatured whey protein before heating than the other ratios and the whey protein denaturation was calculated based on the initial whey protein found in the unheated milk (Table 2). Conversely, the heating time had no significant effect on the whey protein denaturation ($p = 0.1748$), even though the whey protein denaturation appears to be lower after the 1 min treatment for the ratio 1.5:1. Others, such as Remeuf et al. [39], have found an increase in the denaturation of

Table 2 The protein composition of yogurt samples containing different casein:whey protein ratios

Milk base	1.5:1	2.8:1	4.5:1
Protein composition			
Protein (%)	3.70	3.70	3.75
Casein (%)	2.37	2.78	3.21
Whey protein (%)	1.33	0.92	0.54
Casein:whey protein ratio	1.8:1	3.0:1	5.9:1
Soluble whey protein (%)			
After heating at 90 °C×1 min	0.17	0.12	0.15
After heating at 90 °C×4 min	0.05	0.11	0.12
Whey protein denaturation (%)			
After heating at 90 °C×1 min	87.4 a	86.6 a	75.3 b
After heating at 90 °C×4 min	96.3 a	87.8 a	74.2 b

Lines with the same letters are not significantly different at $p < 0.05$

whey of 58 and 77 % after 1 and 5 min at 90 °C, respectively. These levels of denaturation are lower than those we obtained. However, they did not mention the time needed to reach 90 °C, which may vary depending on the heating equipment. In the present study, the heat treatments were performed in batch and a heating duration of 13–15 min was necessary to reach 90 °C, which could account for the higher whey protein denaturation. Moreover, many authors showed that heat treatment had an impact on yogurt viscosity and syneresis, but their treatment lasted longer or higher temperatures (80 °C×30 min; 95 °C×10 min) were used [17, 37]. Others reported that the formation of whey protein–casein linkages induced by a severe heat treatment (90 °C×10 min) increased the yogurt viscosity and water-holding capacity [30, 33, 41]. Additionally, increasing the amount of β -lactoglobulin denaturation, as influenced by the heat treatment, improved the yogurt water-binding capacity; however, when the complex formation between β -lactoglobulin and κ -casein reached a maximum, a further increase in the severity of the heat treatment no longer improved the syneresis [13]. The formation of whey protein aggregates and β -lactoglobulin/ κ -casein complexes upon milk heating were reported to improve the yogurt texture and thus serve as a food ingredient capable of adjusting the yogurt to the desired microstructure [29, 47]. Thus, the increase in the denaturation of whey for the ratio 1.5:1 correlates with the lower syneresis and the higher viscosity due to the presence of whey protein aggregates and β -lactoglobulin/ κ -casein complexes. However, the grainy texture of this yogurt, due to the presence of whey protein aggregates, is not suitable for industrial application but provides a good model to understand the effect of dairy microstructure on protein digestion. In addition, the ratio 2.8:1 presented a nice smooth texture with less syneresis than the ratio 4.5:1.

Because no distinctive differences were observed between both heating times, only the 4 min treatment will be presented for the *in vitro* digestion study of the yogurt samples with different casein:whey protein ratios.

In Vitro Digestion of Yogurts with Different Casein:Whey Protein Ratios

Oral (O2), gastric (G5, G15, G30, G60) and duodenal (D5, D15, D30, D60) digestions were conducted *in vitro* to mimic human digestion. The pH was monitored to ensure no variation occurred during the *in vitro* digestion of each yogurt formulation and no significant differences were observed (data not shown). The average pH was 4.87 ± 0.25 , 3.67 ± 0.14 and 7.09 ± 0.10 at the end of the oral, gastric and duodenal digestion steps, respectively. After each digestion step, the residual amount of yogurt was weighed after centrifugation to measure the yogurt disintegration (Fig. 2a); a lower value indicates a

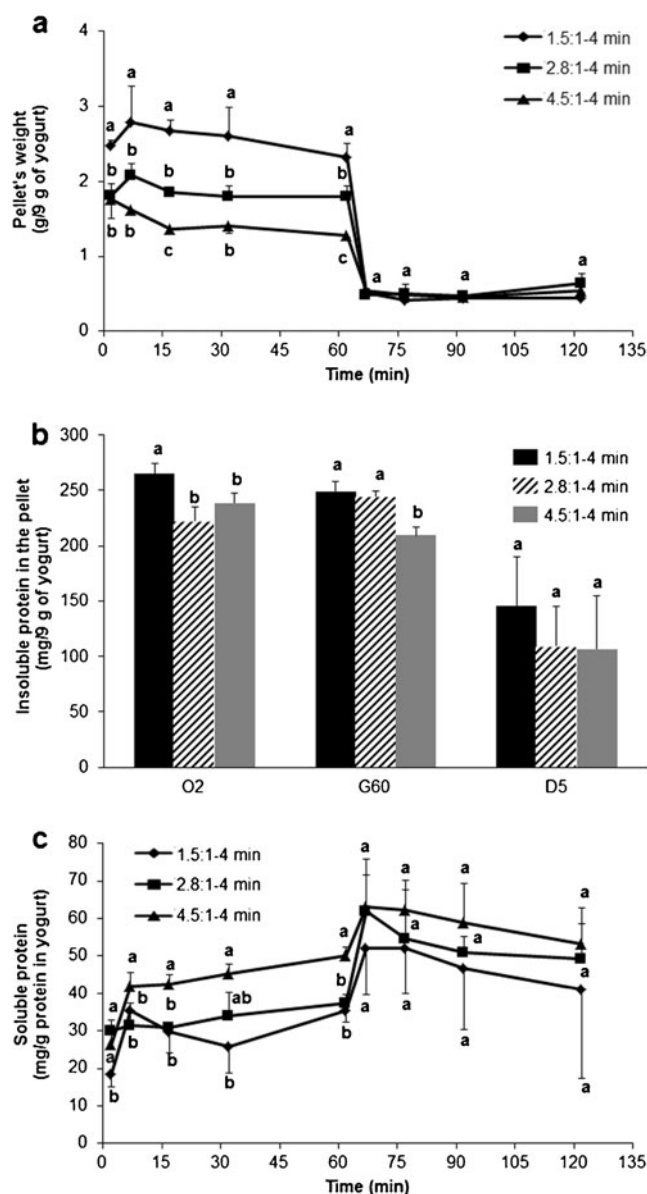


Fig. 2 The effect of the casein:whey protein ratio on yogurt disintegration (a), insoluble (b), and soluble protein release (c), during *in vitro* digestion. Data are presented as the mean $\pm$ SD of three experiments. Identical letters within the same time are not significantly different at $p < 0.05$

higher extent of disintegration. The yogurt samples with a ratio 1.5:1 were significantly less disintegrated during the oral and gastric digestion compared to the other ratios. Besides, the pellet contained significantly more insoluble protein than ratio 4.5:1 (Fig. 2b). These findings could be explained by the higher content of β -lactoglobulin, which is resistant to pepsin hydrolysis during gastric digestion [5, 27]. Additionally, the higher consistency index found for the ratio 1.5:1 might be responsible for the lower yogurt disintegration during the gastric digestion. Viscous meals have been reported to delay digestion through several actions by affecting food mixing and interaction with enzymes, hindering nutrient diffusion and

interacting with the gastric mucosa, resulting in slower gastric emptying [8, 14]. Thus, the release of nitrogenous compounds could be affected. Additionally, the yogurt microstructure seems to be an important aspect; the disintegration remained similar throughout the oral and gastric digestions, suggesting that stirring and pepsin have little impacts on the disruption of yogurt microstructure in vitro. However, after 5 min of the duodenal digestion, the extent of the yogurt disintegration was reduced to the same level and the amount of insoluble protein (Fig. 2b) was similar for each ratio. The dilution due to the addition of the enzymatic solutions and the rapid hydrolysis resulted in similar values for all of the yogurt samples. Thus, it seems that gastric digestion is an important step with regard to yogurt matrix disintegration and we suggest that it is influenced by the yogurt microstructure.

The amount of soluble protein found in the supernatant was significantly higher during the oral and gastric digestions for the ratio 4.5:1, while the difference no longer existed after the duodenal digestion (Fig. 2c). This is in accordance with the yogurt disintegration results. The identification of the soluble protein found in the supernatant was visualized by SDS-PAGE (Fig. 3). After the oral digestion, less β -lactoglobulin was found for the ratio 1.5:1, even though the initial whey protein content was higher compared to the other ratios. This could be explained by a lower soluble whey protein content, which is in agreement with the soluble protein release that is significantly lower for the ratio 1.5:1 (Fig. 2c). Additionally, Beaulieu et al. [3] found that whey protein aggregates and β -lactoglobulin/ κ -casein complexes from heated milk model solutions were precipitated after centrifugation. Furthermore, an SDS-PAGE analysis of the supernatant after centrifugation revealed a decrease in the intensity of the bands compared to the uncentrifuged solutions [3]. This confirms the results obtained in the present study where the 1.5:1 ratio contained less soluble proteins due to the presence of aggregates that have precipitated after centrifugation (Fig. 2b). After 5 min of

gastric digestion, more soluble casein remained for the 1.5:1 ratio compared to the other ratios, which again is linked to the lower disintegration rate of this yogurt matrix (Fig. 3). Moreover, others have suggested that the resistance of casein to in vitro digestion is increased in heated milk due to the casein aggregation observed with the denatured whey protein [15]. Because the 1.5:1 ratio contained large amounts of denatured whey protein, it is possible that the number and size of the aggregates were increased compared to the 4.5:1 ratio, leading to higher amounts of undigested casein. A previous study has shown that heated milk acidified to a pH of 6.0 has a slower clotting rate in the presence of pepsin compared to raw milk, which was related to the increased proportion of denatured β -lactoglobulin [34]. In addition, increasing the amount of whey protein in heated milk resulted in the formation of aggregates with an increased size [37]. It could be possible that those larger size complexes were more hardly digested, thereby limiting the accessibility of casein to pepsin. Additionally, others have reported that the hydrolysis of κ -casein by pepsin was decreased as the amounts of β -lactoglobulin/ κ -casein complexes increased [12]. More work is necessary to attain a better understanding of the mechanisms by which these complexes affect digestion rates.

After the duodenal digestion, the amount of soluble protein released (Fig. 2c) and the intensity of the bands present on the electrophoresis gel (Fig. 3) remained the same for each ratio. The proteins observed on the electrophoresis gel represent the digestive enzymes added during the in vitro digestion (ESM 2). These data strongly suggest that duodenal digestion is fast under the conditions used; this is consistent with the findings of previous studies that have shown similar results with milk using the same in vitro digestion method [23].

The release of free amino acids was measured (Fig. 4) and few differences were observed throughout the oral digestion (data not shown). The total amount of amino acids released after the gastric and duodenal digestions was not different among the ratios (Fig. 4a). However, the total amino acid content sharply increased after the gastric digestion, which also confirms that the duodenal digestion was fast. The branched amino acids were also quantified (Fig. 4b) and their amount increased throughout the digestion time. Additionally, independently of the ratio tested, high levels of leucine (Leu) and lysine (Lys) were found, which is mainly explained by the high level of those amino acids in casein and whey protein (data not shown). Others have observed that casein and whey proteins exhibit different patterns of release of amino acids in humans [6, 18]. Hall et al. [18] observed a significant difference in the concentrations of valine, isoleucine, leucine and threonine after the consumption of casein and whey protein. In the present study, we were not able to see any differences in the release

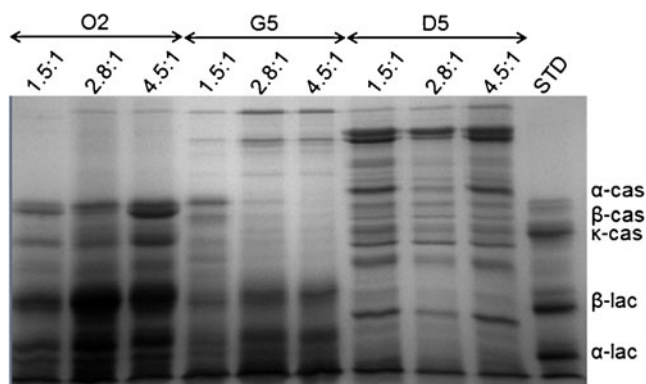


Fig. 3 The SDS-PAGE analysis of soluble protein of the supernatant of digested yogurt samples containing different casein:whey protein ratios. Oral (O2), gastric (G5), and duodenal (D5) samples are shown

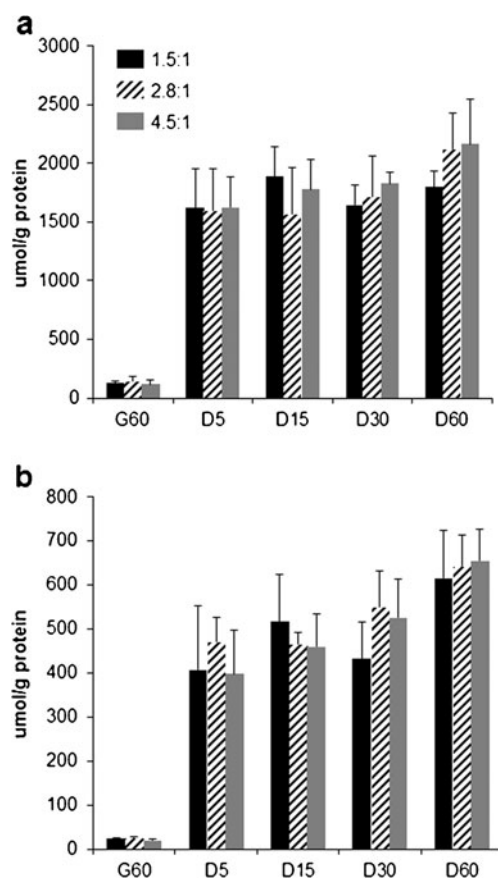


Fig. 4 The total (a) and branched (b) amino acid released after gastric and duodenal digestion of yogurts with different casein:whey protein ratios. Data are presented as the mean $\pm$ SD of three experiments

of those amino acids according to the casein:whey protein ratios used. However, several amino acids found after the *in vitro* digestion (Leu, Trp and Tyr) were reported to be involved in the reduction of food intake [10] or as precursor of serotonin and dopamine [46]. Thus, as proven by others, milk proteins possess valuable nutritional components that are involved in many physiological functions.

The apparent viscosity at the end of each digestion step (O2, G60 and D60) was determined as it has a major impact on gastric emptying and enzyme susceptibility. The viscosity of the digesta will be among other things dictated by protein concentration, ionic force and the presence of insoluble particle/yogurt microstructure. Although the 1.5:1 ratio initially had a higher viscosity (ESM 1), its apparent viscosity after the oral digestion (O2) was lower than the other ratios (Fig. 5a). The presence of the β -lactoglobulin/ κ -casein complex and a higher content of the denatured whey protein aggregates in the 1.5:1 ratio could have caused a sedimentation under shearing and contributed to the reduction of the viscosity of the digesta. This is in agreement with the higher amounts of insoluble protein found for this ratio after oral digestion (Fig. 2b). At the end of the gastric digestion (G60),

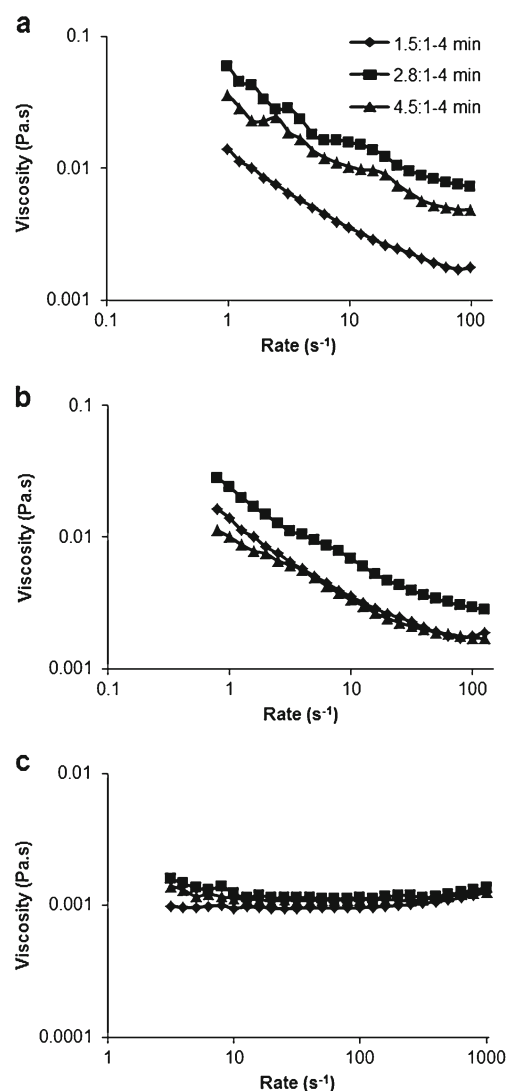


Fig. 5 The viscosity of the yogurt samples with different casein:whey protein ratios after a 2 min oral (a), 60 min gastric (b), and 60 min duodenal (c) digestion

the viscosity of ratio 2.8:1 was higher (Fig. 5b). However, at the end of the duodenal digestion, no difference was observed among all the ratios (Fig. 5c). A curve modelization of the apparent viscosity flow curves was conducted (Table 3). After the oral digestion, the consistency index (k) was significantly higher for the ratio 2.8:1, but no significant difference was observed for the flow behavior index (n). After the gastric digestion, only the zero shear viscosity (η_0) that represents the viscosity at low shear rates was significantly higher for the 1.5:1 and 2.8:1 ratios. This is consistent with the higher amounts of insoluble protein found for ratio 1.5:1 and 2.8:1 (Fig. 2b). The infinite shear viscosity (η_∞), which represents the viscosity at high shear rates, was similar for all of the ratios. Because β -lactoglobulin is not degraded during gastric digestion [5, 27], it could account for the higher viscosity found for the non-digested yogurt at the ratios of 2.8:1 and

Table 3 The flow curve modelization and viscosity comparison of yogurts with different casein:whey protein ratios conducted after each digestion step

Digestion step	Model		1.5:1–4 min	2.8:1–4 min	4.5:1–4 min
Oral (O2)	Power law ^a	<i>k</i>	0.019±0.011 b	0.045±0.005 a	0.026±0.006 b
		<i>n</i>	−0.500±0.038 a	−0.458±0.002 a	−0.392±0.086 a
Gastric (G60)	Carreau ^b	η_0	0.056±0.025 a	0.057±0.007 a	0.027±0.001 b
		λ	3.363±0.630 a	15.833±5.823 a	14.251±12.031 a
		<i>n</i>	8.673±7.456 a	3.723±2.600 a	2.032±1.505 a
		η_∞	0.399±0.084 a	0.576±0.027 a	0.552±0.095 a
Duodenal (D60)	— ^c	31.6	1.050±0.088 a	1.103±0.052 a	1.062±0.037 a
		100.0	1.025±0.075 a	1.123±0.040 a	1.084±0.023 a

Lines with the same letters are not significantly different at $p < 0.05$

^a The correlation coefficient was always greater than 0.92

^b The correlation coefficient was always greater than 0.95

^c Viscosity (mPa s) at a shear rate of 31.6 and 100 s^{−1}

1.5:1. It is possible that if the viscosity would have been measured in the early phase of gastric digestion, a clearer difference in viscosity would have been observed. Future work will involve real-time viscosity measurements during the in vitro digestion to have a better understanding of the impact of the chyme viscosity on the kinetics of protein digestion and the release of amino acids. The preliminary results obtained with an integrated system showed larger viscosity differences within the first 20 min of the gastric digestion (data not shown).

After the duodenal digestion, the apparent viscosity was comparable (Fig. 5c) and the apparent viscosity at the shear rates of 31.6 and 100 s^{−1} was compared for each ratio (Table 3). No significant differences were observed and this finding is not surprising because the duodenal digestion is fast and the dilution induced through the addition of the duodenal juices reduced the viscosity within the rheometer shear limit. Moreover, the viscosity found in the duodenum after protein consumption was not reported to have a crucial impact on nutrient availability. However, the addition of fiber to a diet was reported to influence digesta viscosity in the ileum and consequently nutrient absorption in an animal model [20].

The Impact of β -Lactoglobulin/ κ -Casein Complexes and Denatured Whey Protein Aggregates on Protein Digestion

Our data suggest that the casein:whey protein ratio plays an important role in the digestibility of nitrogenous compounds, which is affected by the formation of β -lactoglobulin/ κ -casein complexes and denatured whey protein aggregates after heat treatment. Others have shown that heating milk with increasing concentrations of whey protein produces increased particle sizes, suggesting the formation

of larger aggregates [37]. Moreover, the high levels of whey protein in milk would lead to the saturation of all of the κ -casein binding sites by denatured whey protein and the formation of whey protein aggregates [3]. This phenomenon was observed at a ratio 1.5:1 by Beaulieu et al. [3]. Thus, the size and the proportion of those complexes and/or aggregates could modify protein digestion. Singh et al. [40] studied the β -lactoglobulin/ κ -casein complex in heated milk and showed that the complete κ -casein gastric digestion was fast (~8 min), while 30 % of the β -lactoglobulin remained after 15 min. Interestingly, when this complex was reduced and carboxymethylated, less than 20 % of the β -lactoglobulin remained after 15 min, showing that the disulfide bonds and complex formations play an important role in this protein digestibility. Additionally, a reduction in the κ -casein hydrolysis by pepsin was reported as the amounts of β -lactoglobulin/ κ -casein complexes increased in heated milk [12]. However, heating β -lactoglobulin alone has increased disulfide bound cleavage due to a change in the conformation that significantly decreases its resistance to gastric and duodenal digestion [38]. Others have shown that the formation of whey protein microaggregates did reduce its digestibility in vitro; however, only duodenal enzymes were used [9]. Thus, the formation of β -lactoglobulin/ κ -casein complexes and denatured whey protein aggregates found in yogurt could contribute to the slow gastric digestion in vitro when higher proportions of whey protein (ratio 1.5:1) were used. Additional work will be necessary to understand how those aggregates and complexes impact protein digestion and if they remain intact during gastric digestion. It is believed that those complexes and aggregates will be relatively stable during gastric digestion because they are formed by disulfide bonding that is known to be stable at a low pH [44]. Additionally, the aggregate and complex sizes are an important aspect to consider because

κ -casein saturated with denatured whey protein would limit pepsin accessibility.

Conclusions

Heating milk for 1 or 4 min does not influence the physicochemical properties of yogurts containing different casein:whey protein ratios in the conditions tested. However, the yogurt physicochemical properties were influenced by the casein:whey protein ratio. The impact of the casein:whey protein ratio on the digestibility of yogurt in vitro and the availability of nitrogenous compounds was evaluated for the first time in this study. Yogurts containing different casein:whey protein ratios (4.5:1, 2.8:1 and 1.5:1) exhibited different behaviors. The ratio 1.5:1 was more hardly digested, possibly due to the presence of the β -lactoglobulin/ κ -casein complex and denatured whey protein aggregates that formed after heat treatment. Both the aggregates and complexes are believed to have a larger size in the ratio 1.5:1, which would affect the activity of pepsin on casein. It can be concluded that the digestion kinetics of nitrogenous compounds are influenced by the viscosity and microstructure of the food matrix and therefore could impact their satiating and nutritional properties.

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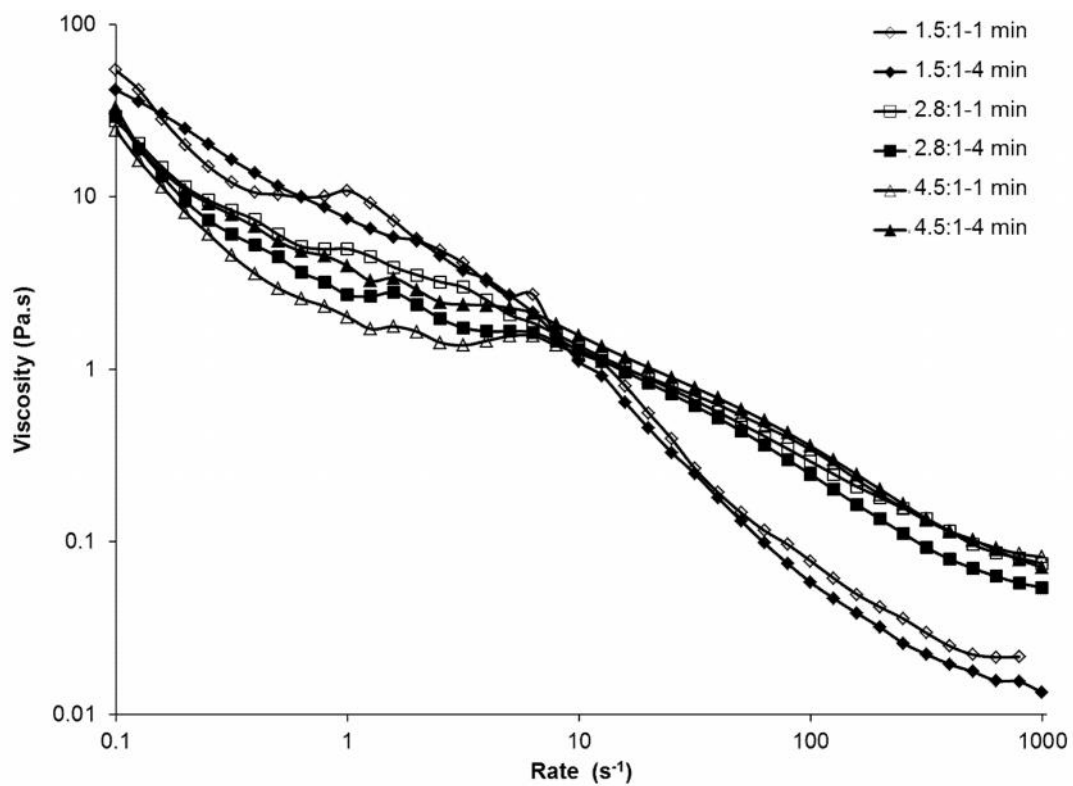
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The ratio of casein to whey protein impacts yogurt digestion *in vitro*

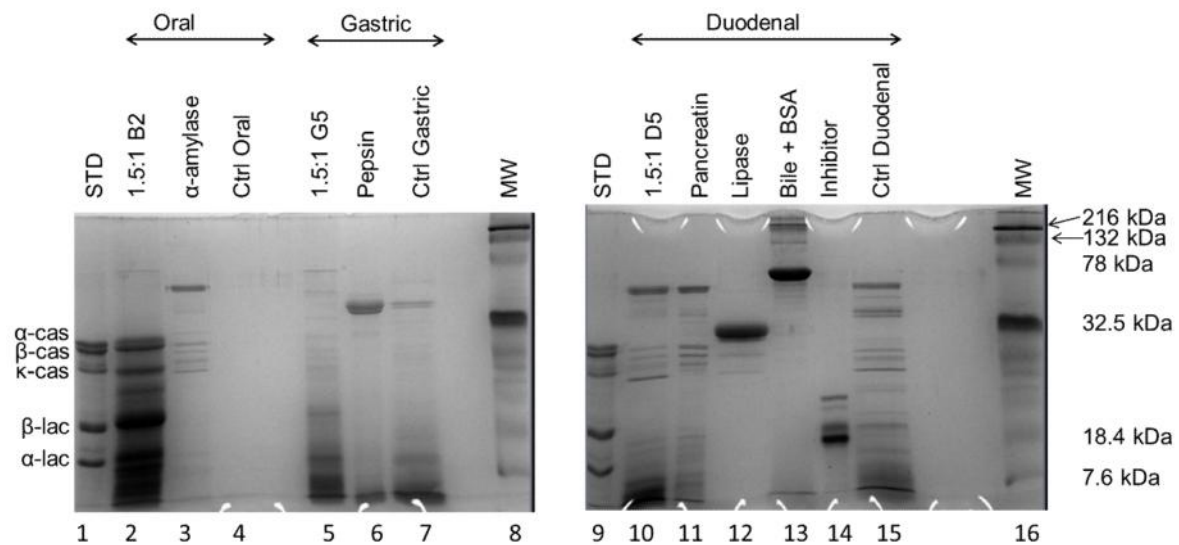
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Online Resource 1 The effect of casein: whey protein ratios and heating times on the viscosity of yogurt



Online Resource 2 The SDS-PAGE analysis of soluble protein of the supernatant of digested yogurt with a casein:whey protein ratio of 1.5:1 after oral, gastric and duodenal digestion. The enzyme used and a water control (Ctrl) digestion for each digestion steps are also shown. The amount of protein for the oral Ctrl sample was too low to be detected on the SDS-Page gel. The amount of proteins loaded in each wells were: 2 µg line1 and 9; 20 µg line 2, 5 and 10; 2.7 µg line 3; 0.3 µg line 4; 7.5 µg line 7, 11-13; 3.6 µg line 14; 15 µg line 7 and 15.