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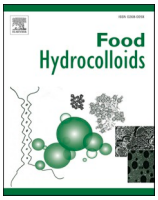

Interplay between microstructure, mechanical properties, macrostructure breakdown and *in vitro* gastric digestion of whey protein gels

Dan Liu [a,*], Anja E.M. Janssen [b], Paul A.M. Smeets [a], Markus Stieger [a]

[a] Division of Human Nutrition & Health, Wageningen University & Research, Wageningen, the Netherlands
[b] Laboratory of Food Process Engineering, Wageningen University & Research, Wageningen, the Netherlands

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ABSTRACT

Gastric digestion of proteins is influenced by multiple factors including microstructure, mechanical properties and structure breakdown during mastication. The interplay between these factors affects protein digestion but is underexplored. This study aimed to investigate the contribution of microstructure, mechanical properties and macrostructure breakdown on *in vitro* whey protein gastric digestion. Whey protein isolate (WPI) was mixed with different types of polysaccharides (κ-carrageenan, ι-carrageenan, pectin) at various concentrations to obtain heat- or acid-induced gels with distinct microstructures (homogeneous, coarse stranded, protein continuous and bi-continuous) and Young's moduli (*E*, 19–165 kPa). Structural breakdown during mastication was mimicked crudely by cutting single gel cylinders into several smaller cubes to increase the total surface area by a factor of 2.65. *In vitro* gastric digestion was measured using the INFOGEST 2.0 protocol with minor modifications. Homogeneous heat-induced WPI/κ-carrageenan gels showed the highest digestion rate followed by protein continuous, coarse stranded and bi-continuous heat-induced WPI/κ-carrageenan gels with similar *E*. A 1.47-fold increase in *E* decreased the digestion rate of acid-induced homogeneous WPI/ι-carrageenan gels by a factor of 0.22. In contrast, a 1.13–1.83-fold increase in *E* barely changed the digestion rate of acid-induced protein continuous WPI/pectin gels. A 2.65-fold increase in total surface area increased the digestion rate of all gels by a factor of 1.35–2.54 depending on microstructure and mechanical properties. We conclude that the microstructure of protein gels affects *in vitro* protein gastric digestion and the impact of Young's modulus on *in vitro* protein gastric digestion depends strongly on the microstructure of protein gels.

1. Introduction

Protein is an essential macronutrient in our daily diets. Understanding protein digestion is very important due to the crucial contribution of proteins to tissue and muscle building. Food digestion involves multiple physical and chemical processes. For solid foods, the macroscopic food structure is physically broken down during oral processing by mastication, leading to the formation of a food bolus that is safe enough to be swallowed. Enzymatic digestion of protein food boli starts in the stomach during the gastric phase (Capuano & Janssen, 2021). In the stomach, the bolus is mixed with gastric juice which contains pepsin, hydrochloric acid, salts and organic substances such as mucins, starting the digestion of proteins. Pepsin breaks down peptide bonds within amino acid chains at low pH resulting in the formation of polypeptides which are further hydrolyzed into oligopeptides, tripeptides, dipeptides and free amino acids by trypsin and chymotrypsin, and are ultimately absorbed by transporters in the intestinal epithelium (Capuano & Janssen, 2021; Kong & Singh, 2008).

Gastric digestion of proteins is influenced by multiple factors including food microstructure, mechanical properties and macrostructure breakdown during oral processing. Protein hydrolysis of soy protein gels was affected by variations of the microstructure. The degree of soy protein hydrolysis was higher for porous, homogeneous gels than for coarser and more aggregated gels although the hardness of the porous, homogenous gels was higher than that of the coarser and more aggregated gels (Zhao, Wu, Chen, Zhao, & Sun, 2020). β-Lactoglobulin gels with fine stranded networks showed higher degree of proteolysis than gels with coarser particulate networks during *in vitro* gastric digestion. It is not clear whether the difference in proteolysis of β-lactoglobulin gels was caused by differences in the microstructure or difference in the mechanical properties as the gels with fine stranded networks were less elastic than the other gels (MacIerzanka et al., 2012). Whey protein gels

* Corresponding author.
E-mail address: dan1.liu@wur.nl (D. Liu).

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with dense agglomerates and low gel strength showed slower simulated gastrointestinal digestion than gels with fine stranded homogeneous networks and high gel strength (Singh, Øiseth, Lundin, & Day, 2014). Luo, Borst, Westphal, Boom, and Janssen (2017) reported that whey protein gels with fine stranded protein networks with smaller pores showed lower amounts of peptides in SGF after *in vitro* gastric digestion than gels with larger pores, although the former was made with higher protein content than the latter. Several studies explored the impact of mechanical properties of protein gels on digestion. Overall, increasing hardness of protein gels decreases gastric digestion. Whey protein gels with higher Young's modulus released less free amino groups in simulated gastric fluid (SGF) during *in vitro* gastric digestion than whey protein gels with lower Young's modulus (Deng, Mars, Van Der Sman, Smeets, & Janssen, 2020). Soft whey protein emulsion gels were emptied faster from an *in vitro* human gastric simulator than hard whey protein emulsion gels caused by higher levels of disintegration during gastric digestion (Guo et al., 2015). Similarly, harder soy protein gels (tofu made with $CaSO_4$) showed lower amino acid content after *in vitro* gastric digestion than softer soy protein gels (tofu made with glucono-δ-lactone) (Lou et al., 2022).

Gastric digestion of proteins is not only affected by food microstructure and mechanical properties, but also by the macrostructure breakdown during oral processing. Macroscopic structural breakdown of solid foods during mastication typically increases the total surface area of the food bolus that is swallowed, providing a larger surface area for enzymatic protein digestion. For various types of foods such as jellies, carrots and breads, it has been reported that with increased hardness, the number of bolus fragments increases and the size decreases (Chen, Khandelwal, Liu, & Funami, 2013; Jalabert-Malbos, Mishellany-Dutour, Woda, & Peyron, 2007; Pentikäinen et al., 2014) leading to an increase in total surface area (How et al., 2021). Increasing the particle size of whey protein gels by a factor of 3.6 after simulated oral processing decreased the *o*-phthaldialdehyde (OPA) reactivity by a factor of 0.4 (Homer, Williams, R., Williams, A., & Logan, 2021). In a computer simulation, the protein digestibility index decreased by a factor of 0.5 at the end of the gastric phase when the average particle size of the meat bolus increased 12 times. This model considered particle size, gastric pH and meat buffering capacity as main factors influencing protein gastric digestion (Sicard, Mirade, Portanguen, Clerjon, & Kondjoyan, 2018). Doubling the chewing time led to the formation of more and smaller particles in the boli of chicken and soy protein-based chicken and increased *in vitro* protein hydrolysis by a factor of 1.16 (Chen, Capuano, & Stieger, 2021). Thus, macrostructure breakdown during oral processing plays an important role in *vitro* gastric protein digestion.

In summary, it has been demonstrated that *in vitro* protein digestion depends on the microstructure, mechanical properties and macrostructure breakdown during oral processing. However, most studies either focused on the effect of mechanical properties on *in vitro* protein digestion without exploring the effect of microstructure on protein digestion (Deng et al., 2020; Lou et al., 2022) or the effect of microstructure on protein digestion was confounded by the effect of mechanical properties on protein digestion (Luo et al., 2017; Singh et al., 2014; Zhao et al., 2020) i.e., microstructure and mechanical properties were varied simultaneously. Most studies concluded that *in vitro* protein digestion was affected by the combined effects of mechanical properties and microstructure. The independent effects of microstructure and mechanical properties of foods on *in vitro* protein digestion and the interplay between microstructure, mechanical properties, macrostructure breakdown and protein digestion remain underexplored.

The aim of this study was to explore the contribution of microstructure, mechanical properties and macrostructure breakdown on *in vitro* gastric digestion of whey protein gels. The rate and degree of *in vitro* whey protein gastric digestion was compared (a) between WPI/polysaccharide gels differing in microstructure with similar mechanical properties (Young's modulus) and surface area, (b) between WPI/polysaccharide gels differing in mechanical properties (Young's modulus) with same microstructure (homogeneous or protein continuous) and surface area, and (c) between WPI/polysaccharide gels differing in surface area with same mechanical properties (Young's modulus) and same microstructure (homogeneous, coarse stranded, protein continuous and bi-continuous). We hypothesized that there is an interplay between microstructure and mechanical properties of WPI/polysaccharide gels during *in vitro* protein digestion and that increasing the surface area of gels promotes *in vitro* whey protein digestion independent of microstructure and mechanical properties.

2. Materials and methods

2.1. Materials

Whey Protein Isolate Bipro™ with 97.9% protein content and 1.9% ash was purchased from Davisco Food International, Inc. (Le Sueur, USA). Food grade κ-carrageenan and ι-carrageenan were kindly provided by CP Kelco U.S., Inc. (Atlanta, USA). Food grade glucono-δ-lactone (GDL) was kindly provided by Roquette, Inc. (Lestrem, France). Vanilla extract (Dr. Oetker, NL) and sweetener (AH Zoetjes, NL containing cyclamate and saccharin) were purchased from a local supermarket (Albert Heijn, Wageningen, NL). Pepsin from porcine gastric mucosa, high-methoxyl pectin (HM pectin, 70–75% degree of esterification) and other chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Preparation of heat-induced WPI/κ-carrageenan gels

Preparation of heat-indued WPI/κ-carrageenan gels was based on Çakir and Foegeding (2011) with minor modifications. κ-Carrageenan concentration and ionic strength were varied to obtain different microstructures. The gel formulations are shown in Table 1. WPI powder was dissolved in 50 mM, 100 mM and 250 mM NaCl solution and stirred for 17 h at room temperature to obtain a 20.6 w/w% WPI stock solution. The pH of the WPI stock solution was adjusted to 7 by addition of 5 M NaOH. κ-carrageenan powder was dissolved in NaCl solutions at twice the final concentration and stirred for 30 min at 90 °C. The κ-carrageenan solutions and WPI stock solution were incubated in a water bath for 15 min at 45 °C. To improve the flavor of the gels for a follow-up

Table 1
Sample name and composition of heat-induced WPI/κ-carrageenan gels differing in microstructure with similar mechanical properties (Young's modulus).

Sample name	Microstructure	Young's modulus(kPa)	Ingredients		
			WPI (w/w%)	κ-carrageenan (w/w%)	NaCl (mM)
Homogeneous gel	Homogeneous	19	10.3	0.0	100
Coarse stranded gel	Coarse stranded	19	10.3	0.1	250
Protein continuous gel	Protein continuous	26	10.3	0.2	50
Bi-continuous gel	Bi-continuous	21	10.3	0.3	50

Note: All gels contained 0.88 w/w% vanilla extract and 0.14 w/w% sweetener. Mean of the Young's modulus (Table 3) is shown to indicate mechanical properties.

study which involved human mastication and sensory evaluation, vanilla extract and sweetener were added to the κ-carrageenan solutions and stirred for 1 min to dissolve. Equal amounts of WPI stock solution and κ-carrageenan solution were mixed while stirring. The pH of the solution was adjusted to 7 by addition of 5 M NaOH and the warm solutions were poured into syringes. Syringes containing the warm solutions were covered with aluminum foil while standing straight being immersed in a water bath. Solutions were heated in the water bath for 30 min at 80 °C to form gels. Syringes containing the heat-induced WPI/κ-carrageenan gels (pH 7) were kept upright at room temperature for at least 1.5 h to cool down. Samples were stored at 4–5 °C and removed from the refrigerator 1.5 h before all measurements.

2.3. Preparation of acid-induced WPI/polysaccharide gels

To obtain whey protein gels with the same microstructure but different mechanical properties, cold-set acid-induced gels were prepared by adding GDL as acidifier (De Jong & Van De Velde, 2007; van den Berg, van Vliet, van der Linden, van Boekel, & van de Velde, 2007). Whey protein isolate was dissolved in water at 12 w/w% for 2 h at room temperature. The solution was heated in a water bath at 68.5 °C for 2.5 h to obtain solutions of whey protein aggregates. HM pectin and ι-carrageenan solutions were mixed with the whey protein aggregate solution to obtain homogeneous gels and protein continuous gels, respectively. The 1 w/w% ι-carrageenan solution was prepared by dissolving ι-carrageenan powder in water at 80 °C for 2 h, then storing the solution at room temperature overnight. Before mixing with whey protein solution, ι-carrageenan solution was heated at 80 °C for 30 min. HM pectin was dissolved in water at 90 °C for 40 min to obtain a 2 w/w% pectin solution. Different amounts of polysaccharide solutions were mixed with the 12 w/w% whey protein aggregate solution to obtain final concentration of 9 w/w% whey protein and ι-carrageenan (0.006 and 0.23 w/w %) or HM pectin (0.05, 0.1, 0.2, 0.3 and 0.4 w/w%) (Table 2). All mixtures were stirred at room temperature for 2 h. GDL was added to the solutions at 0.75 w/w% and stirred for 3 min. Solutions were poured into end-closed syringes and sealed with parafilm and stored at room temperature for 48 h to form gels. The final pH of gels was 4.6. Gels were stored at 4–5 °C and removed from the refrigerator 1.5 h before all measurements.

2.4. Characterization of microstructure using CSLM

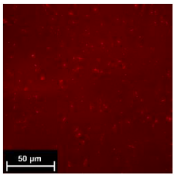
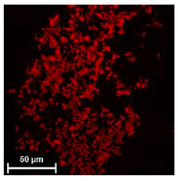
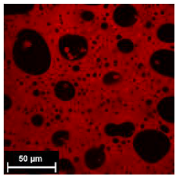
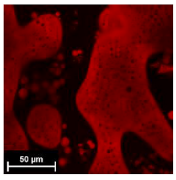
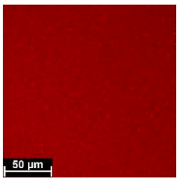
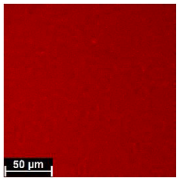
Samples were prepared as described in sections 2.2 and 2.3 except that a solution of 0.002% Rhodamine B was added to 10 mL

Table 2
Sample name and composition of acid-induced WPI/polysaccharide gels differing in mechanical properties (Young's modulus) with homogeneous or protein continuous microstructure.

Sample name	Microstructure	WPI (w/w %)	GDL (w/w %)	ι-carrageenan (w/w%)	Pectin (w/w%)
WPI/ι-carrageenan gel					
81 kPa	Homogeneous	9	0.75	0.006	/
119 kPa	Homogeneous	9	0.75	0.23	/
WPI/pectin gel					
90 kPa	Protein continuous	9	0.75	/	0.05
102 kPa	Protein continuous	9	0.75	/	0.1
127 kPa	Protein continuous	9	0.75	/	0.2
144 kPa	Protein continuous	9	0.75	/	0.3
165 kPa	Protein continuous	9	0.75	/	0.4

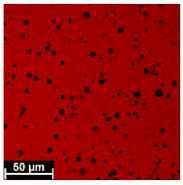
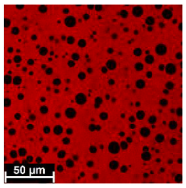
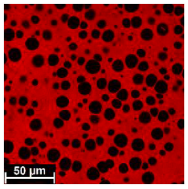
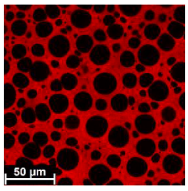
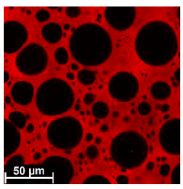
Note: Sample names were given based on the Young's modulus of gels (Table 3).

Table 3
Microstructure and Young's modulus of mixed WPI/polysaccharide gels.

Sample	Young's modulus (kPa)	Microstructure	
Heat-induced WPI/κ-carrageenan gel			
Homogeneous gel	19 ± 2[b]	Homogeneous	
Coarse stranded gel	19 ±1[b]	Coarse stranded	
Protein continuous gel	26 ± 1[a]	Protein continuous	
Bi-continuous gel	21 ± 1[b]	Bi-continuous	
Acid-induced WPI/ι-carrageenan gel			
81 kPa	81 ± 4[b]	Homogeneous	
119 kPa	119 ± 7[a]	Homogeneous	
Acid-induced WPI/pectin gel			

(continued on next page)

Table 3 (*continued*)

Sample	Young's modulus (kPa)	Microstructure	
90 kPa	90 ± 4^{e}	Protein continuous	
102 kPa	102 ± 4^{d}	Protein continuous	
127 kPa	127 ± 5^{c}	Protein continuous	
144 kPa	144 ± 5^{b}	Protein continuous	
165 kPa	165 ± 6^{a}	Protein continuous	

Note: Data (mean ± SD, n = 24) with different superscript letters in same column within subsections are significantly different ($p < 0.05$). The scale bars correspond to 50 μm. The red areas represent the protein-rich phase, and the black areas represent the polysaccharide-rich phase. The composition of heat-induced gels is summarized in Table 1 and the composition of acid-induced WPI/ι-carrageenan gels and acid-induced WPI/pectin gels in Table 2.

polysaccharide/WPI solutions before heat-induced or acid-induced gelation. Gels were manually cut into slides and placed onto carriers. A Zeiss LSM 510-META confocal laser scanning microscope (CSLM) equipped with a He–Ne laser was used. All images were recorded at room temperature. The excitation wavelength was 543 nm, and the emission wavelength was 580 nm. The Zeiss Plan-Apochromat 63x/1.4 oil immersion objective was used to observe microstructure inside gels. Images were snapped when a representative structure was found after widely scanning through the gels. Images were collected with a resolution of 1024 × 1024 pixels and size of 179 × 179 μm or 184 μm × 184 μm.

2.5. Cutting of gels and characterization of mechanical properties

After preparation gels were pushed out of the syringes and cut into cylinders of 10 mm height and 26 mm diameter (total surface area 1880 mm^2) with a slicer equipped with steel-wires. To crudely mimic the macrostructure breakdown during oral processing, these gel cylinders were manually cut with a knife into 31–35, small cubes of around 5 × 5 × 5 mm (total surface area 4650–5300 mm^2, Supplementary Fig. 1). Uniaxial compression tests were performed on the gel cylinders (10 mm height, 26 mm diameter) using a Texture Analyzer (Instron Corp. 5564, USA) equipped with a load cell of 2000 N. Uniaxial compression tests were performed at a compression speed of 1 mm/s to 90% of initial height. The Young's modulus (E, kPa) was extracted from the initial slope of the true Hencky's stress-strain curves within the strain region of 0.05–0.15. Measurements were repeated on eight cylinders per sample and each sample was replicated three times yielding 24 measurements per sample.

2.6. In vitro gastric protein digestion

In vitro gastric protein digestion was performed according to the INFOGEST 2.0 protocol with minor modifications (Minekus et al., 2014). *In vitro* gastric digestion experiments were carried out in triplicates. About 5 g of gel sample either as one cylinder (10 mm height, 26 mm diameter, total surface area 1880 mm^2) or many, small cubes (31–35 cubes of 5 × 5 × 5 mm, total surface area 4650–5300 mm^2) were immersed into 30 mL simulated gastric fluid (SGF, containing 25 mmol/L $NaHCO_3$, 47.2 mmol/L NaCl and 2000 U/mL pepsin, pH = 2) for 3 h at 37 °C under continuous gentle stirring. The pH of SGF was kept constant at 2 by titrating 1 M HCl solution using an automated titrator. Small aliquots of SGF (100–300 μL) were taken once per hour. SGF samples were diluted with water and heated at 90 °C for 5 min while mixing using a pre-heated Eppendorf thermomixer to inactivate pepsin.

Free amino groups were determined in the SGF samples using the OPA method (Nielsen, Petersen, & Dambmann, 2001). Briefly, 10 μL sample solutions were added into 96-plate wells containing 200 μL OPA reagent and mixed by shaking for 3 min. The absorption of mixed solutions was determined by using a microplate photometer (Thermo Scientific 357, USA) at 340 nm. Serine standard solutions (0–200 mg/mL) were used to obtain a standard calibration curve using Milli-Q water as blank. *In vitro* gastric digestion data were average over the triplicate measures. The rate of free amino groups released into SGF during the first 2 h was taken as digestion rate. The mean of free amino groups concentration in SGF after 2 h digestion was taken as final free amino group concentration.

To explore and compare the effects of microstructure, mechanical properties and total surface area on *in vitro* protein digestion, relative changes in digestion rate and final free amino group concentration caused by modifications of the microstructure, Young's modulus or total surface area between gels were calculated as

$$\text{Relative change} = \frac{v(sample)}{v(reference)} \quad (1)$$

with v(*sample*) referring to the value of the digestion rate or value of the final free amino group concentration of a gel sample and v(*reference*) referring to the value of the digestion rate or value of the final free amino group concentration of a reference gel. For example, to estimate the magnitude of the effect of increasing the total surface area from 1880 mm^2 to 5300 mm^2 of a gel with a given microstructure and Young's modulus, the relative change in digestion rate was obtained as Relative change = Digestion rate (gel with 5300 mm^2)/Digestion rate (gel with 1880 mm^2).

To compare the relative impact of microstructure and total surface

area on *in vitro* protein digestion, the relative changes in digestion rate between heterogeneous WPI/κ-carrageenan gels (protein continuous, coarse stranded and bi-continuous) with 1880 mm^2 and 4850–5300 mm^2 surface area were calculated to estimate the magnitude of the effect of total surface area. Relative changes in digestion rate between heterogeneous WPI/κ-carrageenan gels and homogeneous WPI/κ-carrageenan gels with 1880 mm^2 surface area were calculated to estimate the effect of microstructure. To compare the relative impact of Young's modulus and total surface area, relative changes in digestion rate between WPI/ι-carrageenan gels with same Young's modulus (119 kPa) but differing in total surface area (1880 mm^2 and 4850 mm^2) were calculated to show the effect of increasing in surface area. Relative changes in digestion rate between WPI/ι-carrageenan gels with same surface area (1880 mm^2) but differing in Young's modulus (81 kPa and 119 kPa) were calculated to show the individual effect of Young's modulus. To compare the relative impact of Young's modulus and microstructure, relative changes in digestion rate between homogeneous WPI/ι-carrageenan gels and protein continuous WPI/pectin gels but similar Young's modulus (119 kPa and 127 kPa) were calculated to estimate the magnitude of the effect of microstructure.

2.7. Statistical data analysis

Young's modulus was analyzed using One-way analysis of variance (ANOVA) followed by post hoc comparison (Tukey test) using SPSS statistics software (IBM SPSS Statistics Version 28, IBM Corp). Data were expressed as means ± standard deviation. A level of significance of $p < 0.05$ was chosen. *In vitro* gastric digestion data were averaged over triplicate measures and reported as means ± standard deviation.

3. Results and discussion

3.1. Microstructure and mechanical properties of mixed WPI/polysaccharide gels

3.1.1. Heat-induced WPI/κ-carrageenan gels differing in microstructure with similar Young's modulus

Four distinct microstructures were obtained in heat-induced WPI/κ-carrageenan gels (Table 3). According to the degree of microphase separation between the polysaccharide-rich and protein-rich phase and connectivity of both phases, these microstructures were classified as "homogeneous" which showed no microphase separation; "coarse stranded" which showed an isotropic, coarse stranded protein network distributed through the κ-carrageenan phase; "protein continuous" which showed a connected protein network with unconnected spherical κ-carrageenan rich phase pores and "bi-continuous" which showed both connected protein network and connected κ-carrageenan rich phase. Microphase separation was attributed to electrostatic repulsion between whey protein aggregates and κ-carrageenan because both were negatively charged at pH 7 (De Jong & Van De Velde, 2007; Foegeding, Stieger, & van de Velde, 2017; Çakir & Foegeding, 2011). Compared to protein continuous microstructures, increasing κ-carrageenan concentration from 0.2 to 0.3 w/w% enabled the connection of the κ-carrageenan rich phase which led to the formation of bi-continuous microstructures. Depletion interactions between κ-carrageenan chains and whey protein aggregates contributed to microphase separation as well (Croguennoc, Nicolai, Durand, & Clark, 2001; Çakir & Foegeding, 2011). In case of coarse stranded microstructures, the high ion strength (250 mM NaCl) increased the incompatibility between protein aggregates and κ-carrageenan thus a particulate protein network was formed (Çakir & Foegeding, 2011).

Despite having distinct microstructures, these four heat-induced WPI/κ-carrageenan gels showed similar Young's modulus (Table 3) ranging from 19 to 26 kPa. There were no significant differences in Young's modulus ($p > 0.05$) between the homogeneous, coarse stranded and bi-continuous WPI/κ-carrageenan gels. While the protein continuous WPI/κ-carrageenan gel had a significantly ($p < 0.05$) higher Young's modulus than the three other WPI/κ-carrageenan gels, the difference was small (< 7 kPa). All gels were elastic, soft and self-supporting. These four gels displayed distinct microstructures while having similar Young's modulus enabling the investigation of the effect of microstructure on *in vitro* gastric digestion of heat-induced WPI/κ-carrageenan gels independent from the effect of mechanical properties (Young's modulus) on digestion.

3.1.2. Acid-induced homogeneous WPI/ι-carrageenan gels differing in Young's modulus with same microstructure

Both WPI/ι-carrageenan gels displayed homogeneous microstructures (Table 3). This is in agreement with previous studies (De Jong & Van De Velde, 2007) that suggested low ι-carrageenan concentrations have no effect on the microstructure of WPI gels. By increasing the amount of ι-carrageenan from 0.006 to 0.23 w/w%, the Young's modulus increased significantly ($p < 0.05$) from 81 to 119 kPa (Table 3). De Jong and Van De Velde (2007) also observed an increase of Young's modulus in WPI/ι-carrageenan gel with increasing ι-carrageenan concentration. As both ι-carrageenan and whey protein aggregates carry counter ions, the difference in osmotic pressure was high so that microphase separation was inhibited (De Jong & Van De Velde, 2007). These two gels differed in Young's modulus while displaying the same microstructure (homogeneous) enabling the investigation of the effect of Young's modulus on protein digestion in homogenous gels.

3.1.3. Acid-induced protein continuous WPI/pectin gels differing in Young's modulus with same microstructure

Table 3 shows the microstructure of acid-induced WPI/pectin gels. The microstructure of all gels was characterized by a connected protein network with spherical, pore-like, pectin rich inclusions. With increasing pectin concentration from 0.05 to 0.4 w/w%, the number and size of pectin pores increased in the protein continuous network. This finding is consistent with that previously reported by van den Berg et al. (2007). The Young's modulus of the five protein continuous gels ranged from 90 to 165 kPa (Table 3) and differed significantly ($p < 0.05$) between all acid-induced protein continuous WPI/pectin gels. This set of five gels differed in Young's modulus while displaying the same microstructure (protein continuous) enabling the investigation of the effect of Young's modulus on protein digestion in protein continuous gels.

3.2. Effect of microstructure on in vitro gastric protein digestion

Heat-induced WPI/κ-carrageenan gels (surface area 1880 mm^2) with distinct microstructures but similar Young's modulus and same protein concentration were used to investigate the effect of microstructure on gastric protein digestion independent from an effect of Young's modulus (mechanical properties). The free amino group concentration in SGF during digestion of the WPI/κ-carrageenan gels is shown in Fig. 1. The free amino group concentration differed between gels with different microstructures. Homogeneous gels showed the highest digestion rate (5.61 mmol•L^{-1}/h) and final free amino group concentration (11.26 mmol/L) after 2 h digestion (Table 4). Bi-continuous gels showed the lowest digestion rate (2.74 mmol•L^{-1}/h") and final free amino group concentration (5.74 mmol/L) after 2 h digestion (Table 4). The digestion rate and final free amino group concentration after 2 h digestion of the coarse stranded and protein continuous gels (Table 4) were comparable and smaller than for the homogeneous gels but larger than for the bi-continuous gels. These findings indicate that the microstructure of WPI/κ-carrageenan gels effects *in vitro* gastric digestion independent of the Young's modulus (mechanical properties). The homogeneous microstructure benefited proteolysis the most compared to the heterogeneous microstructures (protein continuous, coarse stranded and bi-continuous). These results can be explained by four mechanisms. Firstly, the dense aggregates in the protein-rich phase of heterogeneous

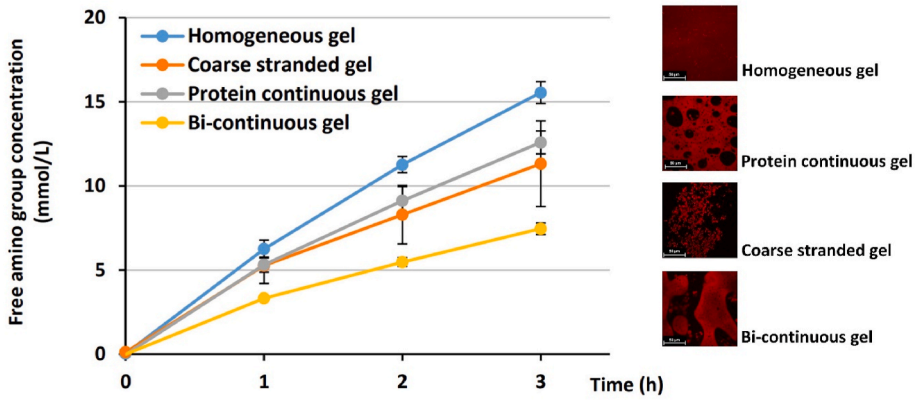


Fig. 1. Free amino group concentration during *in vitro* gastric digestion of heat-induced mixed WPI/κ-carrageenan gels (surface area 1880 mm^2) with different microstructures and similar Young's modulus. Error bars denote standard deviation. The red areas represent the protein-rich phase, and the black areas represent the polysaccharide-rich phase. The scale bars correspond to 50 μm.

Table 4
Digestion rate and final free amino group concentration of mixed WPI/polysaccharide gels (surface area 1880 mm^2) after 2 h *in vitro* gastric digestion. Data are shown as mean ± SD (n = 3).

Sample	Digestion rate (mmol•L^{-1}/h)	Final free amino group concentration (mmol/L)
Heat-induced WPI/κ-carrageenan gel		
Homogeneous gel	5.61 ± 0.21	11.26 ± 0.48
Protein continuous gel	4.57 ± 0.42	9.12 ± 0.83
Coarse stranded gel	4.08 ± 0.86	8.29 ± 1.74
Bi-continuous gel	2.74 ± 0.19	5.47 ± 0.27
Acid-induced WPI/ι-carrageenan gel		
81 kPa	7.03 ± 0.04	14.07 ± 0.08
119 kPa	1.57 ± 0.09	3.12 ± 0.16
Acid-induced WPI/pectin gel		
90 kPa	6.33 ± 0.17	12.64 ± 0.32
102 kPa	7.01 ± 0.10	14.11 ± 0.18
127 kPa	6.70 ± 0.18	13.29 ± 0.53
144 kPa	6.58 ± 0.04	13.05 ± 0.27
165 kPa	7.09 ± 0.18	14.09 ± 0.24

gels might have hindered the proteolysis compared to the loose aggregates in the protein-rich phase of homogeneous gels. This explanation is supported by the study of Singh et al. (2014) who found that whey protein gels with large stranded, heterogeneous microstructures were digested slower than whey protein gels with fine stranded, homogeneous microstructures. They attributed this finding to the denser aggregates of large stranded heterogeneous whey protein gels compared to that of fine stranded homogeneous gels. Secondly, it is reasonable to speculate that in our study more protein molecules were exposed to pepsin at the surface of the homogeneous WPI/κ-carrageenan gels than the heterogeneous WPI/κ-carrageenan gels within the same surface area. The penetration of pepsin into the whey protein gels can be limited to the first 2 mm from the gel surface during *in vitro* gastric digestion (Deng et al., 2020; Luo et al., 2017). This means that proteolysis mainly takes place close to the gel surface during the first hours of *in vitro* protein digestion. For the homogeneous WPI/κ-carrageenan gels, protein molecules were evenly dispersed at the surface, while for the heterogeneous WPI/κ-carrageenan gels, protein molecules were congregated in different locations surrounded by the κ-carrageenan rich phase. Fewer whey protein molecules per unit area might have led to lower proteolysis per unit area for the heterogeneous WPI/κ-carrageenan gels compared to the homogeneous WPI/κ-carrageenan gels. Compared to bi-continuous WPI/κ-carrageenan gels, coarse stranded WPI/κ-carrageenan gels showed a less connected protein phase, i.e., fewer whey protein molecules per unit area at the gel surface but higher free amino acids during *in vitro* gastric digestion. This could be attributed to the rougher surface of coarse stranded gels compared to other heat-induced gels (Supplementary Fig. 2). For the small particles escaped from the rough surface, coarse stranded microstructure together with small particles might facilitated the pepsin migration within the protein network leading to faster and more release of free amino acids to SGF. The third possible explanation could be differences in acid uptake rate and partition coefficient of pepsin between the whey protein gel surface and the SGF. Deng et al. (2020) found higher concentrations of green fluorescent protein which was used to represent pepsin at the surface of whey protein gels with higher swelling ratios. The digestion rate and acid uptake increased with increasing swelling ratio (Deng et al., 2020). We speculate that the gel microstructure of mixed WPI/κ-carrageenan gels is highly related to water migration, acid uptake and pepsin penetration during *in vitro* gastric digestion. The fourth explanation is a potential inhibiting effect of the κ-carrageenan on whey protein digestion, although the concentration of κ-carrageenan used in our study was very low (0.0–0.3 w/w%). Previous studies of milk and whey protein dispersions reported that the addition of 0.5–1.0% alginate decreased the digestion of milk and whey protein by a factor of 0.33–0.63 (Borreani, Llorca, Larrea, & Hernando, 2016; Markussen, Madsen, Young, & Corredig, 2021).

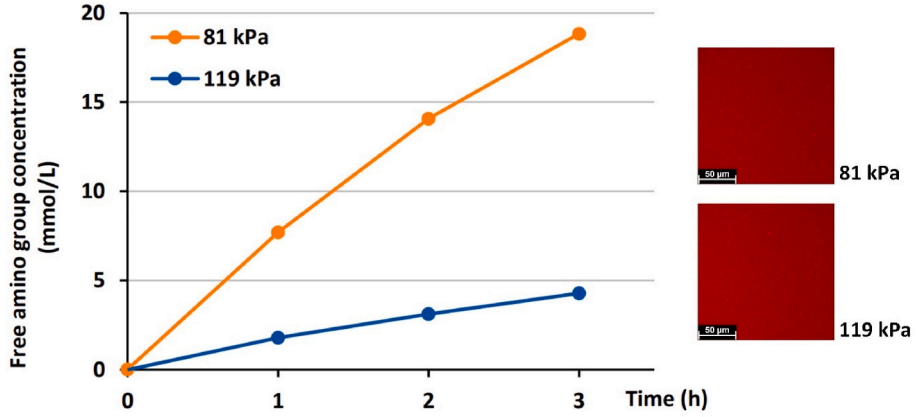


Fig. 2. Free amino group concentration during *in vitro* gastric digestion of acid-induced WPI/ι-carrageenan gels with homogeneous microstructure and different Young's modulus. Error bars are too small to be seen. Only the protein-rich phase is visible in red in CLSM images. The scale bars correspond to 50 μm.

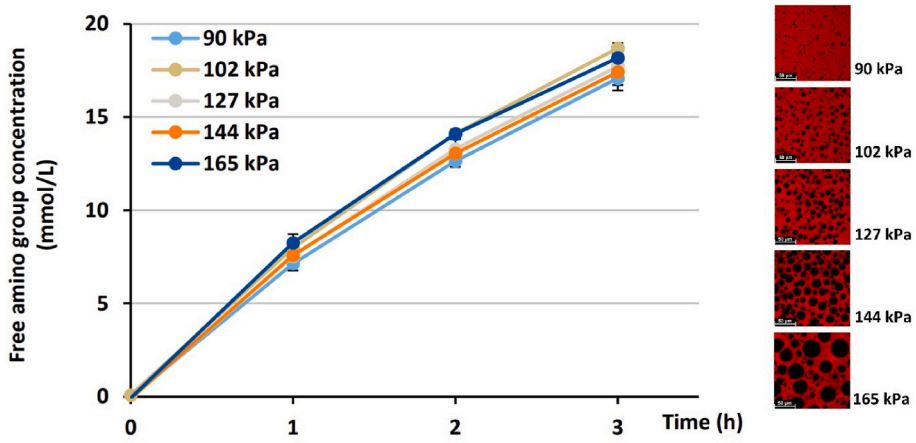


Fig. 3. Free amino group concentration during *in vitro* gastric digestion of acid-induced WPI/pectin gels with protein continuous microstructure and different Young's modulus. Error bars denote standard deviation (n = 3). In the CLSM images, red areas represent the protein-rich phase, and black areas represent the polysaccharide-rich phase. The scale bars correspond to 50 μm.

3.3. Effect of Young's modulus on in vitro gastric protein digestion of homogenous and protein continuous mixed WPI/polysaccharide gels

3.3.1. Acid-induced homogeneous WPI/ι-carrageenan gels

Acid-induced homogeneous WPI/ι-carrageenan gels differing in Young's modulus (Table 3) with same protein concentration (9 w/w%), same microstructure and same total surface area (1880 mm^2) were used to study the effect of Young's modulus on whey protein gastric digestion independent of microstructure. Stiff WPI/ι-carrageenan gels (119 kPa) showed lower free amino group concentrations in the SGF than less stiff WPI/ι-carrageenan gels (81 kPa) during *in vitro* gastric digestion (Fig. 2). After 2 h digestion, the final free amino group concentrations in the SGF were 14.1 mmol/L for less stiff WPI/ι-carrageenan gels (81 kPa) and 3.1 mmol/L for stiffer WPI/ι-carrageenan gels (81 kPa) (Table 4). The digestion rate of the less stiff WPI/ι-carrageenan gel (81 kPa) was 4 times higher than that of the stiffer WPI/ι-carrageenan gel (7.03 vs. 1.57 $mmol \bullet L^{-1}/h$). This demonstrates that whey protein digestion of homogeneous WPI/ι-carrageenan gels can be increased by decreasing the stiffness (decreasing the Young's modulus). These results support evidence from previous studies which suggested that whey protein gels with high Young's modulus inhibited protein hydrolysis (Deng et al., 2020; Guo, Ye, Lad, Dalgleish, & Singh, 2014; Homer et al., 2021).

3.3.2. Acid-induced protein continuous WPI/pectin gels

Similar free amino group concentration profiles (Fig. 3) during *in vitro* gastric digestion are observed for acid-induced protein continuous WPI/pectin gels differing in Young's modulus with same protein concentration (9 w/w%) and same total surface area (1880 mm^2). Increasing the Young's modulus of protein continuous WPI/pectin gels by a factor of 1.83 from 90 to 165 kPa only slightly varied the digestion rate by a factor of 1.12 (Table 4). These findings suggest that the Young's modulus showed only a very limited effect on *in vitro* gastric digestion of WPI/pectin gels with protein continuous microstructure. This outcome is in contrast to the results of homogeneous acid-induced WPI/ι-carrageenan gels (section 3.3.1; Fig. 2) where a 1.47-fold increase in Young's modulus from 81 to 119 kPa decreased the digestion rate by a factor of 0.22 from 7.03 to 1.57 $mmol \bullet L^{-1}/h$. These findings show that the effect of the Young's modulus on protein gastric digestion depends strongly on the microstructure of the whey protein gel. Previous studies suggested that increasing Young's modulus inhibited proteolysis during *in vitro* protein gastric digestion by limiting the concentration of pepsin at the gel surface (Deng et al., 2020; Guo et al., 2014; Homer et al., 2021). However, in the present work, the pore size of protein continuous WPI/pectin gels increased with increasing Young's modulus (Table 3). This might accelerate pepsin diffusion and acid migration from the gel surface inside the gel. We speculate that the combined effect of Young's modulus and pore size led to similar digestion rates and free amino group concentration in the SGF during *in vitro* gastric digestion of acid-induced protein continuous WPI/pectin gels.

The acid-induced protein continuous WPI/pectin gels with Young's

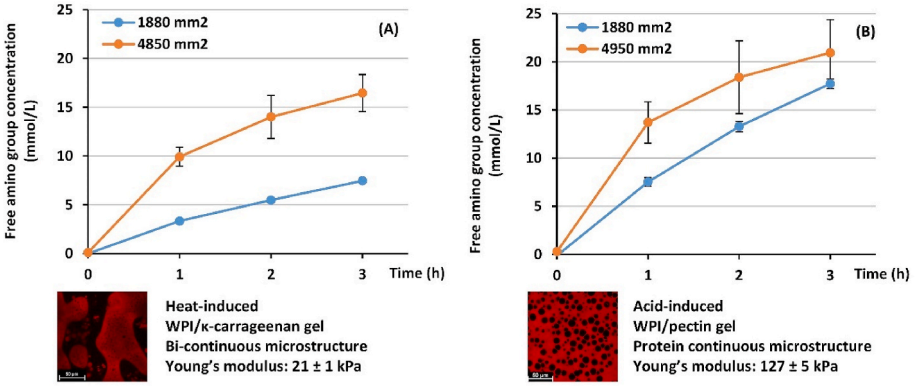


Fig. 4. Free amino group concentration during *in vitro* gastric digestion of mixed WPI/polysaccharide gels (A) with total surface area of 1880 mm^2 and 4850 mm^2 for WPI/κ-carrageenan gels and (B) with total surface area of 1880 mm^2 and 4950 mm^2 for WPI/pectin gels. Error bars denote standard deviation (n = 3). In the CLSM images, red areas represent protein-rich phase and black areas represent polysaccharide-rich phase. The scale bars correspond to 50 μm.

modulus of 127 kPa showed 4.27-fold higher digestion rate (6.70 $mmol•L^{-1}/h$) compared with the acid-induced homogeneous WPI/ι-carrageenan gels with comparable Young's modulus (119 kPa) (1.57 $mmol•L^{-1}/h$). This is inconsistent with the results obtained from heat-induced WPI/κ-carrageenan gels. The protein continuous microstructure increased the whey protein digestion rate of acid-induced WPI/pectin gels, while it decreased that of heat-induced WPI/κ-carrageenan gels. This might be related to the pH, whey protein concentration and polysaccharide type of the mixed gels. Protein concentration and initial pH are main factors affecting gel buffering capacity and degree of protein hydrolysis (Luo, Zhan, Boom, & Janssen, 2018; Mennah-Govela & Bornhorst, 2021; Mennah-Govela, Singh, & Bornhorst, 2019). In our work, acid-induced WPI/polysaccharide gels had 9 w/w% WPI and pH of 4.6, while heat-induced WPI/κ-carrageenan gels had 10.3 w/w% WPI and pH of 7. Moreover, the type of polysaccharide (κ-carrageenan, ι-carrageenan, pectin) might influence whey protein gastric digestion, especially in the protein continuous and bi-continuous gels where the polysaccharide rich phase was concentrated due to microphase separation.

3.4. *Effect of surface area on in vitro gastric protein digestion of mixed WPI/polysaccharide gels*

The free amino group concentration in the SGF during *in vitro* gastric digestion of WPI/polysaccharide gels increased over time and was always higher for all gels with total surface area of 4650–5300 mm^2 compared to 1880 mm^2 (Fig. 4; heat-induced WPI/κ-carrageenan bi-continuous gels and acid-induced WPI/pectin protein continuous gels are shown exemplary). Increasing the total surface area by a factor of 2.65 from 1880 mm^2 to 4650–5300 mm^2 increased the digestion rate to different extents depending on the microstructure of heat-induced mixed WPI/polysaccharide gels (Supplementary Table 1). For gels with similar Young's modulus, increasing the total surface area of homogeneous, protein continuous and coarse stranded WPI/κ-carrageenan gels by a factor of 2.66–2.82 increased digestion rate by a factor of 1.75–1.98, while increasing the total surface area of bi-continuous WPI/κ-carrageenan gels by a factor of 2.58 led to a 2.54-fold increase in digestion rate. For acid-induced homogeneous WPI/ι-carrageenan gels, a similar increase in total surface area (2.64-fold and 2.58-fold) led to higher increase (2.35-fold) in the digestion rate of gels with Young's modulus of 81 kPa compared to the increase (1.55-fold) in digestion rate of gels with Young's modulus of 119 kPa (Supplementary Table 1). An explanation can be that the digestion degree of acid-induced WPI/ι-carrageenan gel with Young's modulus of 81 kPa after cutting was limited by the protein content, since the free amino group concentration in the SGF barely increased during the last hour of digestion (22.02 mmol/L after 2 h and 22.82 mmol/L after 3 h). Similar results were observed for final free amino group concentration. These findings are consistent with those of Mennah-Govela and Bornhorst (2021) who demonstrated that the degree of whey protein hydrolysis and free amino group concentration were higher in smaller gel cubes compared to larger gel cubes after *in vitro* dynamic gastric digestion.

The digestion rate and final free amino group concentration of heat-induced WPI/κ-carrageenan gels were divided by the initial total surface area to exclude the influence of total surface area on protein digestion (Table 5). The digestion rate per mm^2 decreased by a factor of 0.65–0.69 for homogeneous, protein continuous gels and coarse stranded WPI/κ-carrageenan gels. These findings are consistent with those of Mennah-Govela and Bornhorst (2021) who reported higher whey protein hydrolysis per unit area for larger gel cubes (side length of 10.3 mm) compared to smaller gel cubes (side length of 3.1 mm). A possible explanation for this might be the low initial buffering capacity of larger gel cubes with smaller total surface area. The smaller the particle size of the protein gels, the higher the buffering capacity (Mennah-Govela et al., 2019, 2020). A high buffering capacity results in an elevated pH, thereby reducing the protein hydrolysis per unit area (Luo et al., 2018;

Table 5
Digestion rate per mm^2 and final free amino group concentration per mm^2 of heat-induced WPI/κ-carrageenan gels differing in microstructure with similar Young's modulus. The results are expressed as mean ± SD (n = 3).

	Digestion rate/surface area ($\mu mol•L^{-1}•h^{-1}/mm^2$)			Final free amino group concentration/surface area ($\mu mol•L^{-1}/mm^2$)		
	Single, large cylinder (1880 mm^2)	Several, small cubes (4850–5300 mm^2)	Relative change[a]	Single, large cylinder (1880 mm^2)	Several, small cubes (4850–5300 mm^2)	Relative change[a]
Homogeneous gel	2.99 ± 0.11	1.94 ± 0.04	0.65	5.99 ± 0.25	3.84 ± 0.27	0.64
Protein continuous gel	2.43 ± 0.22	1.67 ± 0.07	0.69	4.85 ± 0.44	3.46 ± 0.24	0.71
Coarse stranded gel	2.17 ± 0.46	1.45 ± 0.05	0.67	4.41 ± 0.93	3.17 ± 0.13	0.72
Bi-continuous gel	1.46 ± 0.10	1.35 ± 0.09	0.93	2.91 ± 0.14	2.89 ± 0.49	0.99

[a] Relative change was calculated based on equation (1) (section 2.6). Data from several, small cubes with total surface area of 4850–5300 mm^2 were taken as samples; data from single, large cylinder with total surface area of 1880 mm^2 were taken as reference.

Table 6
Relative changes in digestion rate and final free amino group concentration of mixed WPI/polysaccharide gels during 2h *in vitro* gastric digestion.

Reference	Sample	Relative change[a]	
		Digestion rate	Final free amino group concentration
Microstructure vs. surface area			
Protein continuous gel (1880 mm^2)	Homogeneous gel (1880 mm^2)	1.23	1.23
	Protein continuous gel (5300 mm^2)	1.98	2.01
Coarse stranded gel (1880 mm^2)	Homogeneous gel (1880 mm^2)	1.38	1.36
	Coarse stranded gel (5000 mm^2)	1.83	1.91
Bi-continuous gel (1880 mm^2)	Homogeneous gel (1880 mm^2)	2.05	2.06
	Bi-continuous gel (4850 mm^2)	2.54	2.56
Young's modulus vs. surface area			
119 kPa gel (1880 mm^2)	81 kPa gel (1880 mm^2)	4.48	4.51
	119 kPa gel (4850 mm^2)	2.35	2.38
Young's modulus vs. microstructure			
119 kPa gel (homogeneous)	81 kPa gel (homogeneous)	4.48	4.51
	127 kPa gel (protein continuous)	4.27	4.26

[a] Relative change was calculated based on equation (1) (section 2.6).

Mennah-Govela & Bornhorst, 2021). However, the digestion rate and final free amino group concentration per mm^2 did not change considerably for bi-continuous WPI/κ-carrageenan gels (0.93-fold change in digestion rate per mm^2 and 0.99-fold change in final concentration per mm^2). This result may be explained by the high connectivity of both the WPI and κ-carrageenan rich phase caused by electrostatic repulsion between negatively charged protein aggregates and κ-carrageenan polymers at pH 7 (Foegeding et al., 2017; Çakir & Foegeding, 2011). This lead to the formation of a relatively open microstructure which might have facilitated acid uptake and accelerated local pH decrease (low buffering capacity). Therefore, the potential increase of buffering capacity caused by the increase of total surface area might have been counteracted by a potential decrease of buffering capacity caused by the bi-continuous microstructure. Further studies related to the effect of microstructure on acid uptake ability of WPI gels are needed.

3.5. Interplay between microstructure, mechanical properties, macrostructure breakdown and in vitro gastric digestion of mixed WPI/polysaccharide gels

To compare the relative impact of microstructure and total surface area on *in vitro* protein digestion, heat-induced WPI/κ-carrageenan gels were considered as these gels differed in microstructure while displaying similar Young's modulus (19–26 kPa). For all heterogeneous gels, the relative change in digestion rate caused by increasing the surface area from 1880 mm^2 to 4850–5300 mm^2 was larger than the relative change caused by changing the microstructure from heterogeneous (protein continuous, coarse stranded and bi-continuous) to homogeneous (Table 6). Similar results were obtained for final free amino group concentration (Table 6). These findings demonstrate that increasing the surface area by a factor of 2.62 had a stronger effect on whey protein hydrolysis of WPI/κ-carrageenan gels during gastric digestion than changing the microstructure from heterogeneous (protein continuous, coarse stranded and bi-continuous) to homogeneous. We speculate that *in vitro* gastric digestion of whey protein gels may be influenced more by the gel surface area than the gel microstructure.

To compare the relative impact of Young's modulus and total surface area on whey protein gastric digestion, acid-induced homogeneous WPI/ι-carrageenan gels differing in Young's modulus (81 and 119 kPa) were considered. The relative change in digestion rate (4.48-fold) caused by a 0.68-fold decrease in Young's modulus was more pronounced than the relative change in digestion rate (2.35-fold) caused by a 2.58-fold increase in the surface area (Table 6). Similar results were obtained for final free amino group concentration (Table 6). These findings suggest that *in vitro* gastric digestion of whey protein gels may be influenced more by the Young's modulus of the gels than the gel surface area.

To compare the relative impact of Young's modulus and microstructure on whey protein gastric digestion, acid-induced homogeneous WPI/ι-carrageenan gels and acid-induced WPI/pectin gels were considered. The digestion rate of acid-induced WPI/polysaccharide gels increased by a factor of 4.27 caused by changing the microstructure from homogeneous to protein continuous and by a factor of 4.48 caused by a 0.67-fold decrease in Young's modulus (Table 6). Similar results were obtained for final free amino group concentration (Table 6). This suggests that for acid-indued mixed WPI/polysaccharide gels, changing the microstructure from homogeneous to protein continuous caused similar changes in *vitro* whey protein gastric digestion compared to decreasing the Young's modulus by a factor of 0.67.

These comparisons of the relative impact of microstructure, mechanical properties and surface area on *in vitro* digestion provide an indication of the effect size of these modifications. However, we stress that these comparisons cannot be generalized, as the relative impact on *in vitro* digestion depends strongly on the magnitude of the modification that is applied. For example, if the gel surface area would have been changed by a factor of 10 instead of 2.65, the effect of surface area on *in vitro* protein digestion would probably have been larger and might have exceeded the effect of Young's modulus on *in vitro* protein digestion. Further studies are needed to obtain generalizable conclusions about the relative impact of microstructure, mechanical properties and surface area on *in vitro* protein digestion. Moreover, the interaction between food mechanical properties and oral breakdown should be considered. It has been reported for various types of foods including gels that the harder the foods, the smaller the bolus particle size and the higher the bolus particle number (Chen et al., 2013; Jalabert-Malbos et al., 2007; Pentikäinen et al., 2014), so the larger the total bolus surface area (Goh et al., 2021; How et al., 2021). Therefore, mechanical properties such as Young's modulus of could indirectly affect protein gastric digestion by influencing the total surface area of the bolus when oral processing is involved. Further studies should include oral breakdown when it comes to the effect of mechanical properties on solid food gastric digestion.

4. Conclusions

This study investigated the contribution of microstructure, mechanical properties and macrostructure breakdown on *in vitro* gastric digestibility of whey protein gels. Homogeneous microstructure of mixed WPI/κ-carrageenan gels increased whey protein proteolysis the most followed by protein continuous, coarse stranded and bi-continuous microstructures. The effect of Young's modulus on whey protein hydrolysis of acid-induced gels strongly depends on gel microstructure. Increasing the total surface area facilitated *in vitro* gastric digestion of whey protein gels depending on microstructure. Increasing the surface area by a factor of 2.62 had a stronger effect on whey protein hydrolysis of WPI/κ-carrageenan gels during gastric digestion than changing the microstructure. The mixed WPI/polysaccharide gels provided a practical system to investigate the interplay between microstructure, mechanical properties, macrostructural breakdown and *in vitro* whey protein gastric digestion. The effect of microstructure and its interplay with Young's modulus and total surface area emphasized the importance of the microstructure on whey protein gastric digestion. Further studies should focus on exploring the mechanisms by which the microstructure affects gastric proteolysis, especially the effect of microphase-separated

heterogeneous structures on gel buffering capacity, swelling behavior and partition coefficient of pepsin between the gel surface and the SGF. Moreover, the influence of *in vivo* oral processing on *in vitro* protein digestion should be considered in future studies.

CRediT authorship contribution statement

Dan Liu: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Project administration. **Anja E. M. Janssen:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration. **Paul A. M. Smeets:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration. **Markus Stieger:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2023.109352.

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