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Adding neutral or anionic hydrocolloids to dairy proteins under *in vitro* gastric digestion conditions

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Abstract

The effect of adding uncharged polysaccharides such as konjac glucomannan (KGM) or negatively charged polysaccharides such as alginate to dairy protein ingredients – milk, whey proteins and calcium caseinate – was investigated through simulated *in vitro* gastric digestion. The apparent viscosity, microstructure (light microscopy), particle size distribution and degradation (SDS-PAGE) of the proteins were monitored after different *in vitro* gastric digestion times (0, 30, 60 and 120 min). The addition of KGM increased the viscosity values of the samples during gastric digestion, which probably would increase gastric distention affecting satiety. The microstructure and particle size distribution results showed that the aggregates formed in the dairy protein-konjac glucomannan mixtures at the start of gastric digestion were broken down into smaller ones over time. However, the aggregates formed with the addition of alginate were larger and remained almost unchanged throughout gastric digestion, due to the strong interaction between the opposite charges of the protein and alginate. The SDS-PAGE results showed that whey proteins were more resistant to pepsin digestion than caseins and that the alginate slowed down protein degradation. These findings suggest that a combination of whey proteins and alginate could be used to delay gastric emptying and promote satiety.

Keywords: Dairy proteins; Konjac glucomannan; Alginate; *In vitro* gastric digestion; SDS-PAGE; Microstructure

1 Introduction

Over recent decades, the problems of overweight and obesity have increased and, therefore, the interest in formulating satiating foods has grown. The concept of appetite control comprises two components: satiation (the processes that induce meal termination) and satiety (which determines the intervals between meals) (Geraedts, Troost, & Saris, 2011; Solah et al., 2010). Ingested food evokes satiety in the gastrointestinal (GI) tract by mechanical and humoral stimulation. Post-gastric factors seem to play a key role in satiety through secretion of various peptides by the walls of the small and large intestine in response to ingested food (Geraedts et al., 2011). Satiety signals differ as the meal moves through the gut but include oral (taste and texture), gastric (distension and emptying), and intestinal (distension and nutrient absorption) factors (Hoad et al., 2004). Fibres (carbohydrates resistant to digestion) and various proteins have commonly been used as ingredients in foods and beverages to enhance satiety (Halford & Harrold, 2012).

Proteins suppress food intake, make a stronger contribution to satiety and delay the return of hunger more than fats and carbohydrates (Anderson & Moore, 2004; Geraedts et al., 2011; Solah et al., 2010). The mechanisms by which the peptide products of protein digestion exert their effect on food intake via the gut include slowing stomach emptying and direct or indirect stimulation of gut hormone receptors (Anderson & Moore, 2004). As dairy products contain high levels of protein, they are good for designing satiating food products. Casein is the most abundant milk protein, accounting for 80% of total protein, with whey proteins constituting the remaining 20% (Chen, Chen, & Hsieh, 2016). Hall, Millward, Long, and Morgan (2003) and Veldhorst et al. (2009) found that whey proved more satiating than casein. The digestion and absorption of whey and casein differ in that casein, unlike whey, coagulates in the stomach due to its precipitation by gastric acid. Furthermore, casein is considered a “slow” protein, whereas whey protein is a relatively “fast” protein (Boirie et al., 1997; Veldhorst et al., 2009), so whey consumption leads to higher plasma concentrations of factors known to contribute to satiety, such as amino acids, glucose-dependent insulinotropic polypeptide, glucagon-like peptide-1 and cholecystokinin (Anderson & Moore, 2004; Hall et al., 2003).

A number of studies shows that fibre-rich foods can increase the feeling of satiety and decrease short-term food intake. Certain fibre types bind water and swell, causing increased viscosity, which is associated with delayed gastric emptying

and increased satiety (Halford & Harrold, 2012; Hoad et al., 2004; Peters et al., 2011).

Konjac glucomannan (*Amorphophallus konjac* K. Koch) and alginate are often used to formulate satiating food. Their mechanisms to induce satiation are different due to differences in their charge and structure: konjac glucomannan (KGM) is a neutral polysaccharide while alginate is a negatively-charged polysaccharide.

KGM is a soluble (Fang & Wu, 2004), fermentable and highly viscous dietary fibre (Keithley & Swanson, 2005), due to its high water-absorption capacity (Chua, Baldwin, Hocking, & Chan, 2010). It has a mechanical function in slowing food intake and reducing appetite (Chen, Cheng, Liu, Liu, & Wu, 2006). KGM promotes satiety through the induction of cephalic and gastric-phase signals, delayed gastric emptying and slowed bowel transit time due to the increased viscosity of the gastrointestinal content, and a reduced rate of food absorption in the small intestine leading to attenuated postprandial glucose and insulin surges (Chua et al., 2010).

Alginate gel formation can be triggered by low pH or the presence of divalent cations such as Ca^{2+}. Once the alginate comes into contact with acids in the stomach it can become a gel, leading to prolonged gastric emptying and a considerably slower rate of intestinal absorption (Brownlee et al., 2005). Torsdottir, Alpsten, Holm, Sandberg, and Tolli (1991) found that a small dose of alginate induce delayed gastric emptying. Peters et al. (2011) showed that a specific alginate that gelled strongly in the presence of Ca^{2+} increased satiety more than an alginate that formed a weak gel. Hoad et al. (2004) suggested that a sense of fullness can be obtained by using a palatable, relatively low-viscosity meal (low-G alginate) which forms solids in the stomach, due to distension of the gastric antrum and/or transport of nutrients to the small intestine in the lumps.

Because alginate, KGM and milk proteins are negatively, neutrally and positively charged, respectively, it may be expected that the interaction between the milk proteins and the two hydrocolloids will be different, and therefore, that the protein digestion will also differ. Many interactions can occur between proteins and polysaccharides, depending on the pH and ionic strength of the environment, the ionisation and charge density and the structure and concentration of the different biopolymers. Protein-polysaccharide complexes form due to strong interactions such as covalent bonding, or to several weak interactions (electrostatic, van der Waals', hydrogen or hydrophobic bonding) (Dickinson, 1998; Mouécoucou, Villaume, Sanchez, & Méjean, 2004).

Several authors (El Kossori et al., 2000; Mouécoucou et al., 2004; Polovic et al., 2007; Shah, Atallah, Mahoney, & Pellett, 1982) have reported that the protein digestion rate depends on the chemical composition of the fibres and their physical properties, such as viscosity and protein-polysaccharide complex formation, which provide a physical obstacle at the protein to pepsin enzymatic cleavage site and reduce its activity (Larsen, Wilson, & Moughan, 1994). Therefore, both KGM, due to its viscosity when mixed with water, and alginate, due to its electrostatic interactions with proteins, may be expected to decrease pepsin activity. The rate of proteolysis will be higher or lower depending on the accessibility of peptide bonds to pepsin, so more or fewer peptides will be broken down into smaller ones or even into amino acids, influencing satiety.

The present study aimed to evaluate the effect of adding neutral or charged hydrocolloids on the *in vitro* gastric digestibility of different milk proteins when formulating satiating dairy products. The digested samples were characterised by apparent viscosity, light microscopy (LM), particle size distribution and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2 Materials and methods

2.1 Ingredients

The ingredients used to prepare the samples were skimmed milk powder (Central Lechera Asturiana, Siero, Spain), whey protein concentrate (AVONLAC 482, Glanbia Nutritionals Ltd., Kilkenny, Ireland), calcium caseinate (Fonterra Co-operative Group Ltd, Reference 385, Palmerston North, New Zealand), konjac glucomannan (GLUCOMANNAN 86 TDF, 120 MESH M202, Trades S.A., Barcelona, Spain), sodium alginate (MANUCOL DMF, FMC Biopolymer, Philadelphia, United States) and distilled water.

2.2 Sample preparation

Three protein solutions were prepared by dissolving skimmed milk powder, whey powder or casein powder, respectively, in distilled water. While slowly adding the powder, the water was stirred and heated (50 °C) for 1 h. Two polysaccharide solutions, konjac glucomannan and alginate, were prepared in the same way. Each protein solution was mixed with each polysaccharide solution to obtain six different samples: MK (10% w/w of skimmed milk powder + 0.5% w/w of konjac glucomannan), MA (10% w/w of skimmed milk powder + 0.55% w/w of alginate), WK (10% w/w of whey protein concentrate + 0.5% w/w of konjac glucomannan), WA (10% w/w of whey protein concentrate + 0.55% w/w of alginate), CK (10% w/w of calcium caseinate + 0.5% w/w of konjac glucomannan) and CA (10% w/w of calcium caseinate + 0.55% w/w of alginate). The percentage of KGM was calculated on the basis of the manufacturer's recommendations and the percentage of alginate was selected through a preliminary study to obtain a similar apparent viscosity to that of KGM systems at low shear rates at 37 °C. Three control samples (M, W and C, all without any polysaccharide) were also analysed.

2.3 *In vitro* gastric digestion

The simulation of gastric digestion was performed in a jacketed glass reactor (1 L capacity) maintained at 37 °C in a temperature-controlled circulating water bath with continuous magnetic stirring throughout the test.

The simulated gastric fluid (SGF) consisted of 0.034 M NaCl, with the pH adjusted to 1.2 using HCl 10 N. The SGF (200 mL) was pre-incubated for 5 min with continuous stirring (Zhang & Vardhanabhuti, 2014b) at 300 rpm.

Each sample (200 g) was mixed with simulated gastric fluid. The pH value was reduced to 1.9 (Abdel-Aal, 2008) with HCl 10 N. Pepsin (P7125, pepsin from porcine gastric mucosa, ≥400 units/mg protein, Sigma–Aldrich) was added at a pepsin to protein ratio of 1:250 on a weight basis, in accordance with Zhang and Vardhanabhuti (2014b). The mix was maintained at 37 °C with continuous stirring (650 rpm) for a recommended time of 120 min, which corresponds to a half-gastric emptying (Minekus et al., 2014). Aliquots (28 mL) were withdrawn into a glass beaker containing 22 mL NaOH (0.1 N) to inactivate the enzyme after 0, 30, 60 and 120 min of incubation. The samples were centrifuged at 5000 g for 15 min at 4 °C. The hydrolysed protein content in the supernatant was measured at 280 nm using a UV–visible spectrophotometer (Cecil Instruments Limited, Cambridge, UK).

2.4 Viscosity measurement

The apparent viscosity was determined using a viscometer (Haake ViscoTester VT6R Plus, Thermo Scientific, Walthman, Mass., U.S.A.) equipped with spindle 1, at 10–200 r.p.m., at 37 °C. Measurements were performed in duplicate.

2.5 Light microscopy (LM)

A Nikon ECLIPSE 80i (Nikon Co., Ltd., Tokyo, Japan) light microscope (LM) was used. A 20 μL aliquot of each formulation was placed on a glass slide and observed at 10x magnification (objective lens 10x/0.45 DIC N1 ∞/0.17 WD 4.0, Nikon, Tokyo, Japan). A camera (ExWaveHAD, model no. DXC-190, Sony Electronics Inc, Park Ridge, New Jersey, USA) was attached to the microscope and connected to the video entry port of a computer. The images were captured and stored at 1280 × 1024 pixels using the microscope software (NIS-Elements F, Version 4.0, Nikon, Tokyo, Japan). The software interfaced directly with the microscope, enabling image recording control. Toluidine blue (1%) was used to stain the proteins.

2.6 Particle size measurements

The particle size distributions of the samples were measured by laser light scattering using a Mastersizer 2000 (Malvern Instruments Limited, Malvern, UK) with two laser sources. The instrument was set up as follows: refractive index of proteins: 1.52, refractive index of the dispersion medium (water): 1.33, assumed absorbance of proteins: 0.1. At least two replications were conducted for each sample.

2.7 Extraction of proteins

The preparation of the samples for electrophoretic study was carried out according to the method of Laemmli (1970) with modifications. Each sample was mixed with Laemmli buffer (4.8% (w/v) sodium dodecyl sulphate, 0.1 M dithiothreitol, 0.001 M ethylenediaminetetraacetic acid, 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue and 0.125 M Tris–HCl; pH 6.8) and heated at 100 °C for 5 min, adjusting the protein concentration of the samples to 2 mg/mL.

2.8 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) on a Multiphor II Electrophoresis System (Pharmacia Biotech, Piscataway, USA), using 15% polyacrylamide gels (ExcelGel SDS Homogeneous) at 600 V, 18 mA, 18 W and 15 °C. Of each sample, 8 μL were loaded into the gel.

The protein bands were stained with Coomassie Brilliant Blue tablets (Phastgel Blue R., Pharmacia). De-staining was performed in an aqueous solution of 25% ethanol and 8% acetic acid. The samples were preserved in a solution of 10% glycerol and 7.2% acetic acid.

The high molecular weight calibration kit used as the standard (Amersham GE Healthcare, UK) consisted of phosphorylase b (97000 Da), bovine serum albumin (66000 Da), egg albumin (45000 Da), carbonic anhydrase (30000 Da), trypsin inhibitor (20100 Da) and α-lactalbumin (14400 Da).

Gels were scanned with an ImageScanner III LabScan 6.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and analysed with the ImageQuant TL Image Analysis Software v7.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

3 Results and discussion

3.1 Viscosity measurement

The apparent viscosities of the samples were measured during *in vitro* gastric digestion for 0, 30, 60 and 120 min. In sample M, prepared only with milk, the viscosity values practically remained unchanged during gastric digestion (they varied from 20.5 mPa s at 0 min–18 mPa s at 120 min). The addition of KGM (sample MK) led to higher viscosity values (101.5 mPa s at 0 min), which decreased along digestion to 80 mPa s at 120 min. In contrast, the addition of alginate barely affected the viscosity at gastric level, being 21.0 mPa s at 0 min and 19.0 mPa s at 120 min.

Samples formulated with whey proteins (W, WK and WA) exhibited slightly lower viscosity values than the correspondent milk samples (M, MK and MA). The values of viscosity kept the same tendency than in milk samples: the addition of KGM in sample WK led to higher viscosity values, which decreased along digestion from 88 mPa s at 0 min–55 mPa s at 120 min, and the addition of alginate did not affect the viscosity.

In the case of samples C and CA, the viscosity values were even lower than the correspondent samples elaborated with milk -M and MA- and with whey protein -W and WA-. In sample CK, the addition of KGM also led to high viscosity values (710 mPa s at

0 min), which decreased at min 30 (516 mPa s) and remained practically unchanged along *in vitro* digestion.

Regardless the protein used, the samples prepared with KGM had the highest apparent viscosity values and the samples prepared with alginate or without polysaccharides presented similar viscosity values. Several authors reported that some soluble fibres bind water and swell, causing increased viscosity and thereby increasing gastric distention, which is associated with delayed gastric emptying and increased satiety (Halford & Harrold, 2012; Logan, Wright, & Goff, 2015; Schroeder, Gallaher, Arndt, & Marquart, 2009). KGM has been found to have volumetric effects, creating a sense of fullness while slowing down physiological processes associated with food digestion (Marcano, Hernando, & Fiszman, 2015). Therefore, the addition of KGM to the milk proteins studied in our work would delay gastric emptying giving place to an increased satiety.

3.2 Light microscopy

The microstructure of the nine samples was monitored by LM at different moments of the simulated *in vitro* digestion (Figs. 1–3). The LM results were correlated with the macroscopic appearance of the samples (Fig. 4).

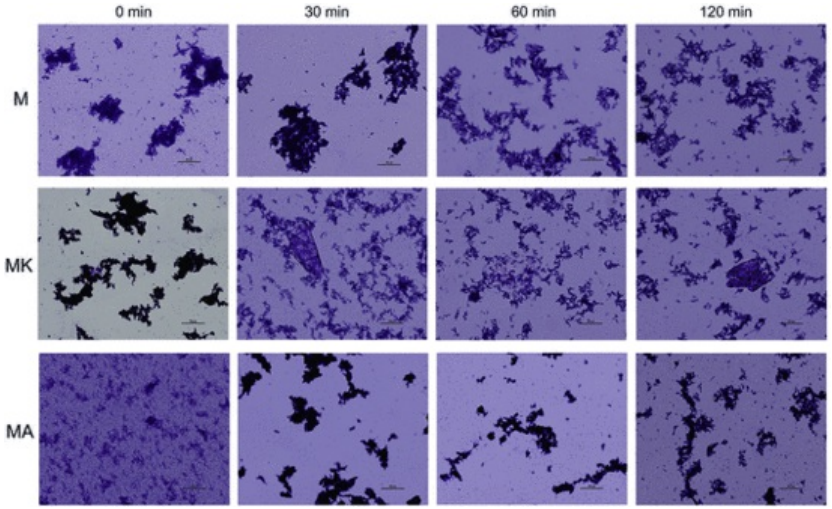

Fig. 1 Light microscopy. Blue toluidine staining. Magnification 10x. Bar 100 μm. Evolution of milk protein microstructure without and with hydrocolloids during *in vitro* gastric digestion at 0, 30, 60 and 120 min. M: milk; MK: milk with konjac glucomannan; MA: milk with alginate.

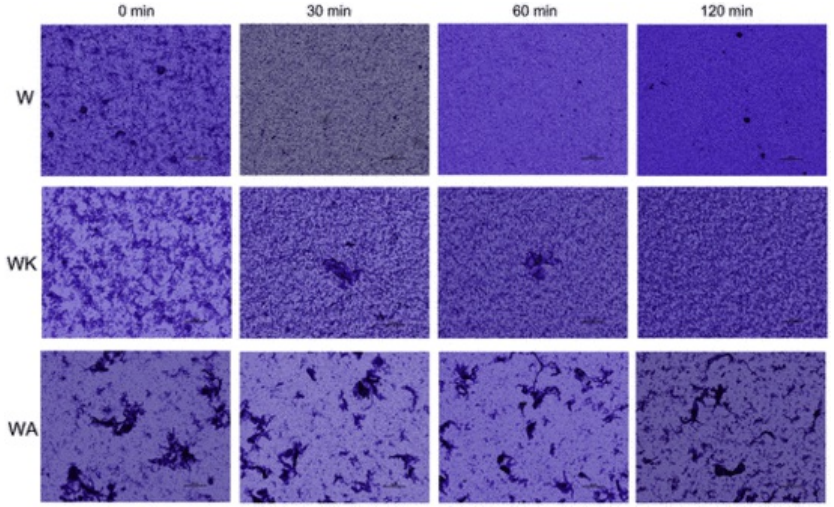

Fig. 2 Light microscopy. Blue toluidine staining. Magnification 10x. Bar 100 μm. Evolution of whey protein microstructure without and with hydrocolloids during *in vitro* gastric digestion at 0, 30, 60 and 120 min. W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate.

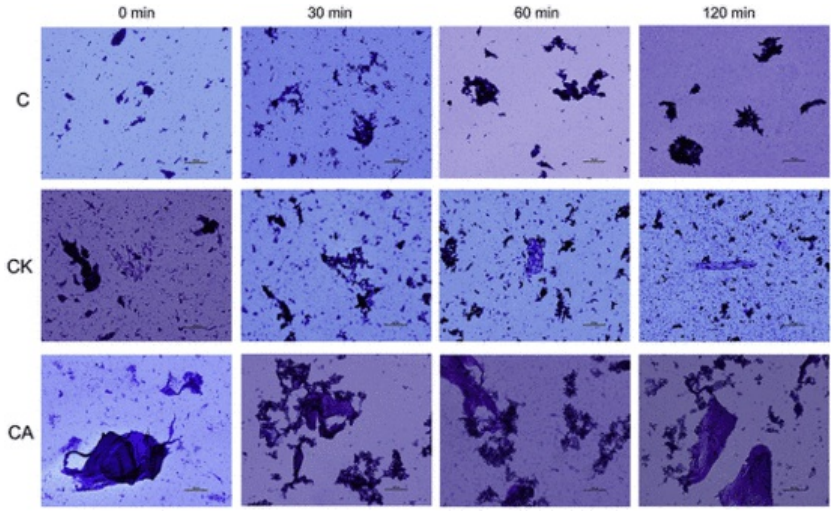

Fig. 3 Light microscopy. Blue toluidine staining. Magnification 10x. Bar 100 μm. Evolution of calcium caseinate microstructure without and with hydrocolloids during *in vitro* gastric digestion at 0, 30, 60 and 120 min. C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate.

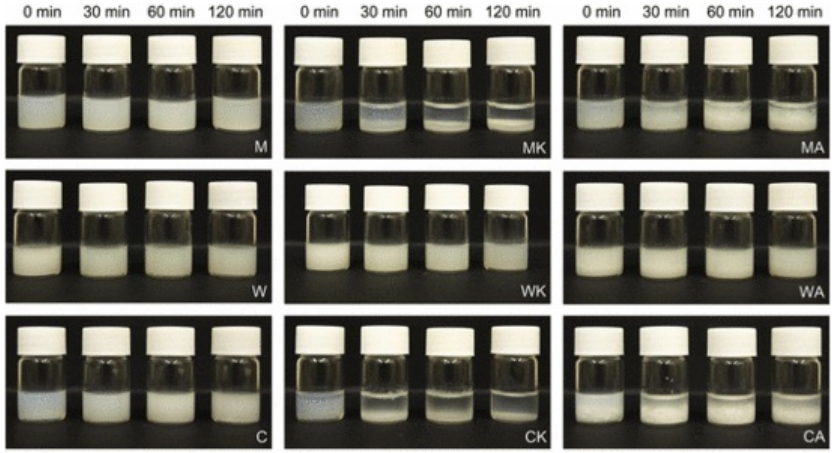

Fig. 4 Macroscopic appearance after 24 h of samples digested for 0, 30, 60 and 120 min. M: milk; MK: milk with konjac glucomannan; MA: milk with alginate; W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate; C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate.

Fig. 1 shows the microstructure of the samples prepared with milk (samples M, MK and MA). The protein (sample M) or protein-polysaccharide polymers (samples MK and MA) are stained purple. In sample M, the aggregates formed at 0 min became slightly larger at 30 min and then showed a gradual degradation of the protein network as the digestion time progressed. Nevertheless, no visual changes were observed at a macroscopic level (Fig. 4). Similar results were obtained during *in vitro* gastric digestion of dairy proteins by Nguyen, Bhandari, Cichero, and Prakash (2015). They observed that at the start of the gastric digestion, the dairy protein (caseins and whey proteins) was clustered into large aggregates, but after 1 h of proteolysis in the stomach these large aggregates of milk protein had become smaller compared to those at 0 min. In sample MK, the aggregates formed at 0 min broke down at a constant rate up until 120 min. Swollen konjac glucomannan particles were observed within the protein aggregates at all the digestion times, showing that no important interactions took place between the protein and the polysaccharide. This was reflected by lower turbidity and a separation of phases (Fig. 4). In contrast, sample MA showed aggregation of the network from 0 to 30 min and no structural changes were observed from 30 min onwards. This can be seen in Fig. 4 as the emergence at 30 min of gelled particles which remained unchanged up to 120 min, and is in accordance with a study by Hemar, Tamehana, Munro, and Singh (2001), who observed that the addition of xanthan gum led to protein aggregation and subsequent phase separation in skim milk powder using confocal scanning laser microscopy.

The samples prepared with whey proteins (samples W, WK and WA) are shown in Fig. 2. The protein network was observed to be finer in the samples with whey proteins than in the milk samples. Samples W and WK showed a similar trend in the degradation of the protein network: slight degradation from 0 to 30 min and no visible changes afterwards. As in sample MK, isolated particles of Konjac were detected in sample WK. The microstructure of samples W and WK corresponded to their macroscopic appearance, where a very slight decrease in turbidity was appreciated (Fig. 4). However, sample WA presented large aggregates due to the strong bond formed between the alginate and the whey proteins, which remained almost unchanged as the digestion time progressed. In accordance with the LM results, no visual changes were observed in sample WA at macroscopic level (Fig. 4). This was also observed by Hemar et al. (2001) in whey protein isolate-xanthan mixtures: the protein aggregates were larger when xanthan was added to whey protein isolate (WPI) than in WPI without xanthan, and they did not observe phase separation in WPI-xanthan mixtures. Agreeing with these results, Zhang, Zhang, and Vardhanabhuti (2014) observed that the protein aggregates in WPI-carrageenan gels formed large clusters, which was likely to be because of the strong attraction between protein aggregates and carrageenan.

Fig. 3 presents the microstructure of samples prepared with calcium caseinate (samples C, CK and CA). Unlike samples M and W, which tended to degrade during digestion, it was observed that sample C tended to aggregate. This was also noticeable in the macroscopic appearance of sample C, where a slight increase in turbidity was observed (Fig. 4). In contrast, in sample CK, the aggregates formed at 0 min showed gradual degradation up to 120 min. The presence of konjac particles seems to limit polymer interactions, decreasing protein coagulation. Hence, the microstructure of CK showed a similar trend to sample MK and also displayed a similar appearance at macroscopic level (Fig. 4). The phase separation in the MK and CK mixtures is likely to be due to flocculation of casein micelles by depletion mechanisms, as observed by Syrbe, Bauer, and Klostermeyer (1998). Sample CA, like samples MA and WA, remained practically unchanged. Large pieces of alginate linked to caseinate could be observed, which are seen in Fig. 4 as gelled particles. Similarly, Hemar et al. (2001) observed “thread-like” dark objects composed of individual strands in confocal micrographs of sodium caseinate-xanthan mixtures.

3.3 Particle size distribution

The particle size measurement was used to quantify the degradation rate of the aggregates formed during digestion (Fig. 5).

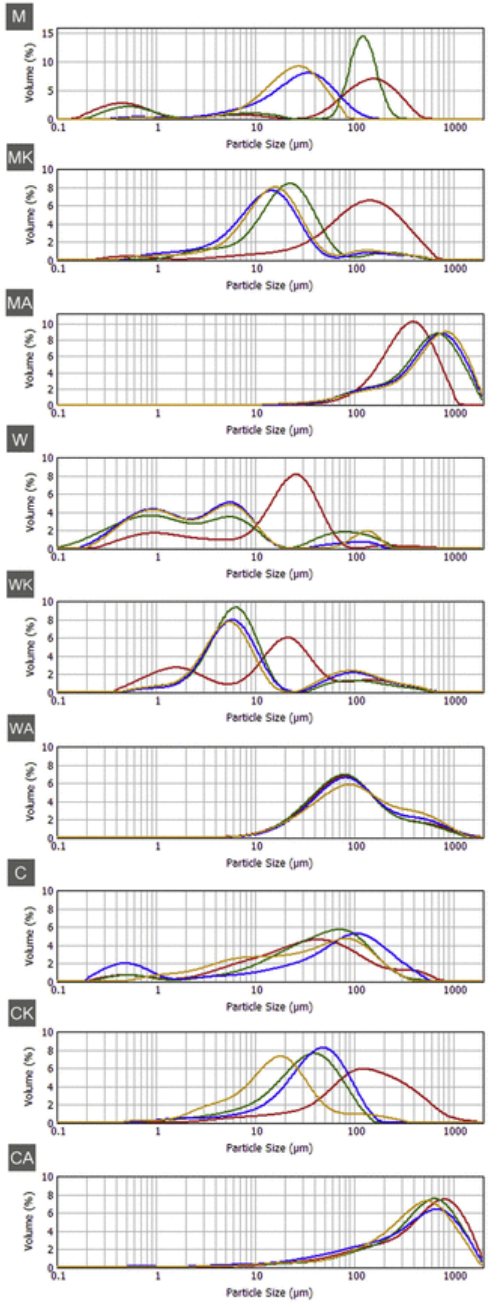

Fig. 5 Particle size distribution of the samples digested for: 0 min (red), 30 min (green), 60 min (blue) and 120 min (yellow). M: milk; MK: milk with konjac glucomannan; MA: milk with alginate; W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate; C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

When the samples prepared with milk (samples M, MK and MA) were analysed, sample M showed aggregates with a particle size between ~35 and 500 µm with a peak at ~170 µm at 0 min, and between ~50 and 260 µm with a peak at ~130 µm at 30 min.

From 30 to 60 min, the particle size decreased considerably, to ~30–40 μm, and from 60 to 120 min it decreased slightly, to ~25–30 μm. This decreasing particle size was also observed by Nguyen et al. (2015) in infant dairy formulations during *in vitro* digestion. In the case of sample MK, the aggregates found at the different gastric digestion times had similar particle sizes to sample M, except at 30 min, where the peak value was lower (~25 μm). For sample MA, the aggregates formed at 0 min were larger (between ~40 and 2000 μm) than those of M and MK. The particle size in sample MA increased from 0 to 30 min and remained practically unchanged afterwards, with a value of ~700 μm. The formation of large aggregates is due to the strong interaction between the protein and the alginate. These results are in accordance with the LM observations, where aggregation of the protein network was observed in the first 30 min in the case of sample MA, whereas samples M and MK showed protein degradation.

When the samples were prepared with whey proteins (samples W, WK and WA), the particle size of the aggregates was smaller than that of the milk samples. The aggregates were also seen as a finer network when observed by LM. Like the samples prepared with milk, samples W and WK had similar particle sizes but those of sample WA were bigger due to the strong protein-alginate interactions. In sample W, the particle size of the aggregates at 0 min was between ~0.25–90 μm, with a peak at ~25 μm. From 30 to 120 min, the particle size distribution became bimodal (one peak at ~0.8 μm and another at ~5.5 μm). In the case of sample WK, the particle size was practically the same as in sample W at 0 min and from 30 to 120 min the particle size distribution shifted from bimodal to monomodal (only one peak, at ~5.5 μm). The particle size of the aggregates in sample WA over the digestion time was between ~10 and 1500 μm, with a peak at ~80 μm. The presence of big aggregates of similar size that remained unaltered over the digestion time was also observed by Zhang and Vardhanabhuti (2014b) when studying the interaction between whey protein isolate and another charged polysaccharide, pectin.

The particle size of samples prepared with caseinate (samples C, CK and CA) was more similar to that of the milk samples than the whey ones, because casein is the most abundant milk protein. Nevertheless, the particle size of sample C increased as the digestion time advanced, rising from ~40 μm to 100 μm (maximum peaks) between 0 and 60 min, although the particle size decreased slightly, to ~90 μm, at 120 min. In sample CK, the particle size decreased from ~120 μm to 17 μm over the digestion time, following a similar trend to sample MK. As in samples MA and WA, sample CA showed large aggregates, but in sample CA the size decreased slightly over the 120 min digestion time, from ~800 μm to 500 μm.

The particle size results matched the LM results in all the samples. Overall, the addition of KGM induced practically no modification of aggregate particle size compared to the respective control samples (samples M, W and C), and a decrease in the particle size was observed over the digestion time. However, the presence of alginate increased the particle size of the aggregates and no important changes were observed during digestion.

3.4 SDS-PAGE

The *in vitro* gastric digestion patterns of the nine samples prepared with different dairy proteins and hydrocolloids were examined using the SDS-PAGE technique under reducing conditions. Fig. 6 presents the electrophoregram, showing the protein profiles before *in vitro* pepsin digestion (0 min) and after different times of simulated *in vitro* digestion (30, 60 and 120 min).

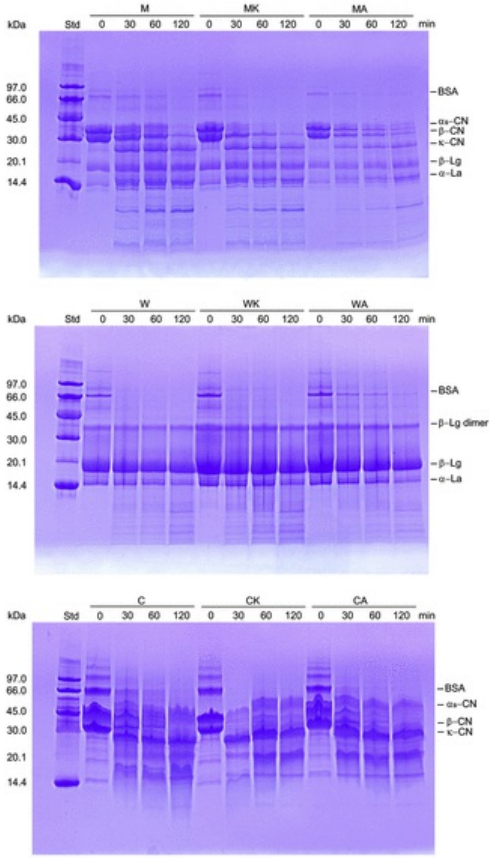

Fig. 6 SDS-PAGE analysis of the samples digested for 0, 30, 60 and 120 min. Std: Standard marker; M: milk; MK: milk with konjac glucomannan; MA: milk with alginate; W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate; C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate.

The samples showed similar proteolysis patterns during simulated gastric digestion, with peptide bands appearing below the α-lactalbumin (α-La) band (14.4 kDa) at 30 min. In the electrophoregram, the hydrolysis products can be seen to be more intense with prolonged digestion times.

In the milk sample without hydrocolloids (sample M), peptides were observed immediately after 30 min and their intensity increased from 10.93% at min 0–22.83% at min 120, while the α_S-casein (including α_{S1}-CN and α_{S2}-CN) and β-casein (β-CN) bands lost intensity, especially at 120 min. The band of α_S-CN lost more intensity (from 28.98% at 0 min–0.71% at 120 min) than the band of β-CN (25.04% and 7.7% at 0 and 120 min, respectively). In agreement with these results, Dupont et al. (2010) and Nguyen et al. (2015) found that bands corresponding to caseins disappeared during digestion of skim milk powder and whey protein isolate-casein mixtures, respectively. The bovine serum albumin (BSA) band also lost intensity during gastric digestion, being 7.42% at 0 min, 3.7% at 30 min, 1.89% at 60 min and 0% at 120 min. However, the band at the κ-casein (κ-CN) molecular weight became more intense at 30 min due to the degradation of caseins by pepsin action. The proteolysis pattern of sample MK was very similar to sample M, agreeing with the particle size and LM results, the only difference being the greater degradation of the α_S-CN and β-CN bands at 30 and 60 min. For example, the intensity of α_S-CN band in sample M was 28.98% at 0 min, 13.42% at 30 min and 11.65% at 60 min; however in sample MK it decreased from 26.96% to 6.24% and to 1.13%, at respectively times. For sample MA, there was less degradation of the α_S-CN and β-CN bands until the 120 min profile compared to samples M and MK. In the case of α_S-CN band, in samples M and MK, the intensity percentages were 28.98% and 0.7% (0 and 120 min, respectively), but in sample MA, the intensity varied from 31.68% to 9%. Furthermore, the peptide bands were much less intense than in samples M and MK (at min 120, the intensity of peptides bands was 28.83%, 21.93% and 13.71%, for sample M, MK and MA, respectively). This could mean that the presence of alginate decreases the digestibility of protein. Chater, Wilcox, Brownlee, and Pearson (2015) found that alginate is able to interact with and disrupt the catalytic mechanism of pepsin, which leads to a reduced level of protein digestion.

In the case of the W samples (samples W, WK and WA) the bands corresponding to β-lactoglobulin (β-Lg), β-Lg dimer and α-La remained practically unchanged during digestion. These results have also been observed by other researchers. Mouécoucou,

Frémont, Villaume, Sanchez, and Méjean (2007) found that the β-Lg dimer, α-La and β-Lg were poorly digested by pepsin in samples prepared with β-Lg/polysaccharide (gum arabic, low methylated pectin or xylan) mixtures. In different infant dairy formulations, Nguyen et al. (2015) observed that β-Lg and α-La completely resisted proteolysis by pepsin during the duration of digestion in the stomach. Other authors have also stated that β-Lg is resistant to pepsin digestion (Mouécoucou et al., 2004; Zhang & Vardhanabhuti, 2014a, b; Zhang et al., 2014). It has been suggested that the acid stability of β-Lg could result in increased internal hydrogen bonding between either two titrated carboxyl groups or one amide and one carboxyl group, reducing the accessibility of its cleavage bonds to the enzyme (Mouécoucou et al., 2004). Faint peptide bands were observed in the electrophoregrams. This could be related to poor digestion of the β-Lg dimer, β-Lg and α-La by pepsin. Moreover, in samples W and WK, the BSA band disappeared completely within 30 min, whereas in sample WA, the BSA band was still observed as digestion progressed (20.04%, 7.43%, 5.22% and 2.41% at 0, 30, 60 and 120 min, respectively). In sample W, the peptide bands appeared discretely at 30 min (10.31%) and their intensity was higher at 120 min (27.30%). In sample WK, the peptide bands were more intense at 30 min (18.45%) and gained further intensity at 60 and 120 min (28.37%). As in the case of sample MA, the intensity of the peptide bands of sample WA was much weaker than those of W and WK: the intensity of peptides bands at min 120 was 27.3% for sample W, 28.37% for sample WK and 17.13% for sample WA. Zhang et al. (2014) reported that the decrease in the intensity of the peptide bands indicates a decrease in digestion rate with time. Once again, this could suggest that the presence of alginate decreased the digestibility of protein.

In the caseinate samples (with and without hydrocolloids), high molecular weight bands (bands above 45 kDa) were present initially but gradually broke down during digestion. In sample C, the α_S-CN and β-CN bands lost intensity from 0 to 30 min (from 13.54% ~~to~~and 17.9% at min 0–11.1% and 9.96% at min 30, respectively) and remained almost unchanged afterwards. Moreover, the bands that appeared below 30 kDa (including the κ-CN band) gained intensity at 30–120 min (53.36% and 64.89% at 30 and 120 min) compared to 0 min (14.05%), due to the degradation of caseins into molecules of lower molecular weight. In sample CK, similar results were observed: degradation of the α_S-CN and β-CN bands and the appearance of bands below 30 kDa. In sample CA, the α_S-CN and β-CN bands lost intensity slightly during digestion. For example, in the case of β-CN, the intensity of the band was 14.02%, 4.43%, 1.25% and 0.61% at 0, 30, 60 and 120 min, respectively. As in samples C and CK, the bands below 30 kDa became more intense from 30 (60.7%) to 120 min (80.11%). No peptide bands were observed on the electrophoregram of the caseinate samples (C, CK and CA). When urea was used for casein extraction, peptide bands could be seen at the same molecular weight as in the milk samples (M, MK and MA) (results not shown).

The whey bands (sample W) were less degraded than the caseinate bands (sample C), which is likely to be due to the fact that whey proteins (WP) remain soluble in the stomach as an intact protein. As Lundin, Golding, and Wooster (2008) stated, the folded structure of β-Lg inhibits enzyme access to potential cleavage sites and, consequently, the protein is considerably more resistant to hydrolysis by pepsin. As a result, WP empties rapidly and is further hydrolysed by pancreatic proteases, being more distally absorbed than casein (Mahé et al., 1996). In contrast, clotted casein is more exposed to gastric peptic hydrolysis – the open structure of proteins in caseinate allows enzymes greater access to target residues, resulting in rapid proteolysis (Lundin et al., 2008) – and empties slowly from the stomach in the form of degraded products, which are subsequently hydrolysed by pancreatic proteases and absorbed in the upper part of the intestine (Mahé et al., 1996).

4 Conclusions

The effect of adding neutral or charged hydrocolloids on the *in vitro* gastric digestibility of different milk proteins was investigated. According to the results, whey proteins remained practically undigested over the digestion time, which is known to be related to rapid gastric emptying. In contrast, caseins formed clusters in the acidic gastric environment and were gradually degraded, therefore a slow emptying of the stomach may be expected.

When formulating satiety products fortified with dairy proteins, polysaccharide-protein interactions have to be taken into account. Regardless of the protein used, its digestion is affected less by the presence of the neutral polysaccharide (KGM) than by the presence of the charged one (alginate). The addition of KGM led to high values of apparent viscosity during gastric digestion, which probably will increase gastric distention and therefore, will affect satiety. However, the strong interaction between alginate and dairy proteins at the gastric pH gave place to large aggregates, which led to limited pepsin access to the protein during gastric digestion. Few hydrolysis products were observed, indicating a significant slowing down of the protein digestion rate, mainly in the case of the whey protein-alginate mixture. These results suggest that this combination could be used to delay gastric emptying and promote satiety.

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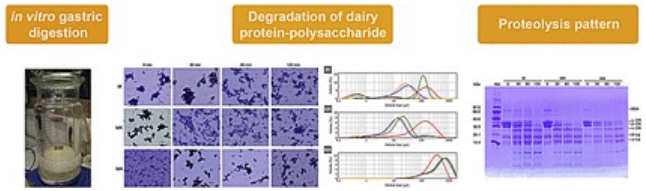
Graphical abstract



Highlights

- Samples with whey proteins remained practically undigested during gastric digestion.
- Samples with caseins were gradually degraded by pepsin during gastric digestion.
- Dairy protein digestion was less affected by the neutral polysaccharide.
- There was a strong interaction between dairy proteins and alginate.
- The addition of alginate slowed down the protein digestion rate.

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