



Monitoring the effect of consumption temperature of full-fat milk on in vitro gastric digestion using Magnetic Resonance Imaging

Conor J Fitzpatrick, Maja Musse, Jiajun Feng, Guylaine Collewet, Tiphaine Lucas, Mark Timlin, Sylvain S. Challos, Stephane Quellec, Didier Dupont, André Brodkorb, et al.

► To cite this version:

Conor J Fitzpatrick, Maja Musse, Jiajun Feng, Guylaine Collewet, Tiphaine Lucas, et al.. Monitoring the effect of consumption temperature of full-fat milk on in vitro gastric digestion using Magnetic Resonance Imaging. Food Hydrocolloids, 2024, 152, pp.109864. 10.1016/j.foodhyd.2024.109864 . hal-04464979

HAL Id: hal-04464979

<https://hal.inrae.fr/hal-04464979v1>

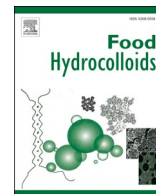
Submitted on 19 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License



Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion using Magnetic Resonance Imaging

Conor J. Fitzpatrick^{a,b,c}, Maja Musse^d, Jiajun Feng^e, Guylaine Collewet^d, Tiphaine Lucas^d, Mark Timlin^a, Sylvain Challos^d, Stephane Quelled^d, Didier Dupont^e, André Brodkorb^a, Daniela Freitas^a, Steven Le Feunteun^{e,*}

^a Teagasc, Food Research Centre, Moorepark, Fermoy, P61 C996, Co. Cork, Ireland

^b School of Food and Nutritional Sciences, Room 240 Food Science Building, University College, Cork, Ireland

^c VistaMilk SFI Research Centre Teagasc Moorepark, Fermoy, Co. Cork, P61 C996, Ireland

^d INRAE, OPALE, 35044, Rennes, France

^e INRAE, Institut Agro, STLO, 35042, Rennes, France

ARTICLE INFO

Keywords:

INFOGEST

MRI

Caseins

Stomach

Lipids

Aggregation

ABSTRACT

This study employed Magnetic Resonance Imaging (MRI) to assess the impact of the consumption temperature of full-fat milk (4 °C, 37 °C, and 60 °C) on its behaviour during *in vitro* gastric digestion. Using the INFOGEST semi-dynamic protocol, and replicating human gastric temperature profiles for cold, warm and hot beverages, it was observed that consuming milk at 4 °C delayed protein coagulation compared to 37 °C and 60 °C by more than 5 min 3D-MRI lipid quantitative analyses showed that fat-rich particles tended to float to the top of the digesta in a process similar to creaming with the 4 °C and 37 °C milks, a phenomenon that was not observed with the 60 °C milk. The quantities of released proteins and free primary amines in the digesta supernatant, indicative of pepsin activity, showed no significant variation with milk temperature. These findings highlight the influence of consumption temperature on the structural reorganization of whole milk during gastric digestion and prompts further inquiry into the potential implications of milk temperature on nutrient delivery into the small intestine.

1. Introduction

During gastric digestion, bovine milk separates into liquid and solid phases because casein micelles coagulate, while the other milk proteins, termed whey proteins, remain soluble. Physiologically, the coagulation of caseins in the stomach is thought to be for better control of the release of proteins from the gastric to the intestinal phase, governing their further breakdown and absorption (Soop et al., 2012). Indeed, gastric digestion plays an important role in the rate of appearance of nutrients in the bloodstream by modulating the emptying of nutrients into the small intestine. Among the factors that may influence the gastric digestion of bovine milk, the kinetics of gastric acidification is important to consider. The pH increases rapidly following the consumption of a neutral pH meal, such as milk, and then decreases gradually throughout digestion down to pH ~2 due to the secretion of acidic gastric fluid (Clark et al., 1993). As during yogurt making, this decrease in pH contributes to the coagulation of casein micelles. It is also essential to enable

proteolytic and lipolytic activity in the gastric compartment. Pepsin is the only proteolytic enzyme present during the gastric phase. Although pepsin is generally considered to have an activity optimum at approximately pH 2 (Piper & Fenton, 1965), it can hydrolyze caseins over a broad range of pH (Salelles, Floury, & Le Feunteun, 2021) and induce their coagulation in a similar way to rennet. For instance, *in vitro* studies with porcine pepsin have shown that this enzyme can hydrolyze 73% of κ -casein at pH 6.3 in 10 min at an activity level of only 2.75 U of pepsin/mL (Yang et al., 2022). This clearly indicates that, for milk, gastric proteolysis may begin straight after the consumption of a meal, even if the pH is close to neutral levels. Therefore, during the gastric digestion of milk in humans, both pH-driven and enzymatic-induced coagulation of casein micelles are at work.

Another factor that may influence the gastric coagulation of caseins, often overlooked, is the effect of the milk consumption temperature. *In vivo* studies have shown that the consumption of a hot or cold beverage can move gastric temperature away from 37 °C for up to 30 min

* Corresponding author.

E-mail address: steven.le-feunteun@inrae.fr (S. Le Feunteun).

<https://doi.org/10.1016/j.foodhyd.2024.109864>

Received 16 November 2023; Received in revised form 2 February 2024; Accepted 5 February 2024

Available online 5 February 2024

0268-005X/© 2024 Elsevier Ltd. All rights reserved.

(McArthur & Feldman, 1989; Sun, Houghton, Read, Grundy, & Johnson, 1988). Moreover, it has also shown that the consumption of liquid foods at different temperatures can affect physiological responses, such as gastric emptying time. For example, it has been observed that beverages consumed at 60 °C tend to exhibit faster gastric emptying times compared to cold liquid meals or beverages (Fujihira, Takahashi, Shimamura, & Hayashi, 2022; Mishima et al., 2009). Studies on the effect of consumption temperature of bovine milk on digestion are limited, however. One recent study investigated the effect of the ingestion temperature of milk on *in vitro* digestion by examining differences in κ -casein hydrolysis when skimmed milk is consumed at different temperatures using *in vitro* semi-dynamic digestion (Yang et al., 2023). This study showed that the initial rate of hydrolysis of κ -casein by pepsin was higher with a 50 °C milk compared to 37 °C and 4 °C milks. However, at the end of digestion, the overall hydrolysis of κ -casein was lowest in the 50 °C milk. This study also suggested that milk consumed at 4 °C may be digested more easily due to the formation of looser and softer curds when compared to milk consumed at 37 °C or 50 °C. Several studies have also investigated the effect of temperature on the rennet-induced milk coagulation. This is of interest as rennet, a mixture of chymosin and pepsin, functions in a similar way to pepsin alone, cleaving κ -caseins, reducing casein micelle repulsion and allowing coagulation to occur. It has been shown that the incubation temperature of milk with rennet affects both coagulation time and curd firmness, with faster coagulation times and harder curds being formed at higher temperatures, which may indicate a similar occurrence in the gastric phase of milk digestion (Nájera A. DeRenobales & Barron, 2003).

As well as protein digestion, lipid digestion is also initiated in the gastric phase. Most lipids in bovine milk exist within the milk fat globule as triacylglycerides (TAGs), with phospholipids and cholesterol mostly present on the surface of the milk fat globule membrane. The breakdown of these lipids begins upon the action of gastric lipase, with 10–30% of lipid hydrolysis occurring in this phase (Hamosh, Bitman, Wood, Hamosh, & Mehta, 1985). Gastric lipase has a stereo-specificity for the sn-3 position of TAGs and, despite having an optimal activity at pH 4–5.4, it is active over a wide range of pH (2–7) (Sams, Paume, Giallo, & Carrière, 2016). Milk fat has been shown to cream during the gastric phase of digestion. This is thought to occur due to enzymatic hydrolysis of proteins located on the surface of the milk fat globular membrane, leading to a destabilisation of the milk fat globules (Ye, Cui, & Singh, 2011). Therefore, apart from the pH, variations in any other conditions susceptible to influencing pepsin activity in the early stages of gastric digestion, such as the meal temperature, have the potential to influence this digestive process.

To better understand the effect of the consumption temperature of milk on its gastric behaviour and digestion under physiologically relevant conditions, *in vitro* digestion is a useful tool that can be combined with many analyses such as microscopy, chromatography, and rheology. While these are all powerful techniques, they are also invasive, making it impossible to study digestive processes without some level of disruption to the mechanism under study. Conversely, MRI is a dynamic and non-invasive imaging technique, which has been used to monitor the digestion of foods and beverages, including milk, both *in vitro* and *in vivo*. A variety of MRI imaging protocols has been recently proposed to monitor gastric digestion *in vitro*, providing insights into structural and chemical transformations of foods. For example, Deng, Seimys, Mars, Janssen, and Smeets (2022) used MRI relaxation parameters to predict the release of protein into the supernatant during semi-dynamic digestion of protein gels, while Chemical Exchange Saturation Transfer (CEST) and Magnetization Transfer (MT) was used to study the milk protein breakdown with MRI (Mayar, Smeets, van Duynhoven, & Terenzi, 2023). Musse et al. (2023) also showed that MRI can be used to monitor the disintegration of solid food particles using ultra-short echo time (UTE) during semi-dynamic *in vitro* digestions of a cheese and bread meal and also quantitatively assess the hydrolysis and creaming of lipids using gradient multi-echo (GRE) for separation of water and lipid

signals.

In continuation of those studies, the objective of this article was to evaluate by MRI the effect of consumption temperature (4 °C, 37 °C and 60 °C) of full-fat milk on its behaviour during semi-dynamic *in vitro* gastric digestion. The term ‘consumption temperature’ will be used throughout this study for the sake of clarity and simplicity, even though it is based on an *in vitro* approach. The milk consumption temperatures we investigated were selected to reflect the likely temperatures at which a human will consume milk. 4 °C was chosen to mimic the consumption of milk directly out of the fridge, 60 °C milk was chosen to mimic the consumption of a hot cup of milk, and 37 °C milk was chosen to allow an experiment where no gastric temperature change occurs. Furthermore, full fat milk was chosen over skim milk upon the hypothesis that milk fat could behave differently depending on the consumption temperature, and to take full advantage of the capabilities of MRI. MRI experiments were designed to assess the formation of the milk curd, the disintegration kinetics of the casein particles upon pepsin action, and to quantify their creaming kinetics. All MRI-related findings are compared to chemical analysis results and discussed in relation to the effect of temperature on digestive enzyme activities and milk coagulum properties.

2. Materials and methods

2.1. Materials

Whole Milk Powder (WMP) was obtained from Moorepark Technology Limited (MTL), Teagasc Food Research Centre, Moorepark Fermoy, Co. Cork, Ireland. This WMP was produced from cows fed a grass-based diet during mid-lactation in Fermoy, Co. Cork, Ireland. Briefly, bulk tank milk was standardised to 0.95 true protein:fat ratio. The standardised milk was pasteurised and homogenised, and subsequently evaporated to 45% solids using single stage falling film evaporator at 65 °C (Anhydro F1 Lab, Copenhagen, Denmark). The milk was then spray dried using a single stage drier with nozzle atomizer, air inlet temperature 180 °C, air outlet temperature 85 °C (Anhydro Spray Dryer, SPX Flow Technology Denmark A/S, Søborg, Denmark). In addition to common analytical grade reagents used in the digestion experiments and sample analysis, rabbit gastric extract (RGE) from Lipolytech (RGE 15, Zone Luminy Biotech Enterprises, France) was also used in the digestion experiments. The activity of pepsin and lipase in the RGE used was 300 U/mg and 10.2 U/mg, respectively, as measured from the assays described in Brodkorb et al. (2019).

2.2. Experimental design

In this study, we investigated four experimental conditions simulating the digestion of full-fat milk consumed at 37 °C in the absence of enzymes (no-enzyme control condition), and the digestion of milk consumed at 37 °C (temperature control condition), at 4 °C, and at 60 °C in the presence of enzymes.

2.3. Milk preparation

WMP was analysed as per Timlin et al.. WMP contained 26.7% protein, 28.2% fat and 38.5% carbohydrates. The evening before use, WMP was reconstituted in osmosis water at 15% (w/v) at room temperature for 5 min before heating to 45 °C for 30 min under magnetic stirring. Milk was then stored overnight (max 12 h) at 4 °C to ensure complete rehydration. Rehydrated full-fat milk contained 4.0% protein, 4.2% lipid, and 5.8% carbohydrates.

2.4. Semi-dynamic gastric *in vitro* digestion

The gastric phase of the semi-dynamic INFOGEST protocol (Mulet-Cabero, Egger, et al., 2020) was used with some modifications to enable the reproduction of gastric temperature curves observed *in vivo* as

further described below (McArthur & Feldman, 1989; Sun et al., 1988). All digestions were carried out in triplicate using RGE as the source of lipase and pepsin.

2.4.1. Preparation of digestive fluids

Simulated salivary fluid (SSF 1.25x concentrated, pH 7.0, no amylase) and simulated gastric fluid (SGF 1.25 × concentrated, pH 1.5) were prepared according to Brodtkorb et al. (2019). RGE was added as a separate solution, prepared by adding RGE to water at activity levels of 80,000 U of pepsin and 2400 U of lipase/mL.

2.4.2. Digestion protocol

To allow optimum temperature control, reconstituted full-fat milk (20 mL) was set to the required temperature (see section 2.4.3.) and added onto the basal secretions already contained in the gastric digestion jacketed vessel (ref. 6.1418.150, Metrohm, Ireland): 1.458 mL of SSF, 10.2 µL of $\text{CaCl}_2(\text{H}_2\text{O})_2$, 1.645 mL of SGF, 0.109 mL of RGE (or water for the no enzyme control experiments) and 0.790 mL of water. This gastric digestion vessel, which was connected to a cryo water bath to enable the reproduction of temperature curves observed in human studies (see section 2.4.3.), was then placed in the centre of the MRI scanner. Due to the constraints of conducting the experiments in an MRI scanner, the addition and stirring of digestive fluids throughout the gastric phase were performed manually. More specifically, SGF and enzyme should have been added at a rate of 0.2808 mL/min and 0.0148 mL/min, respectively, according to Mulet-Cabero, Egger, et al. (2020). In this experiment, SGF was added using a stepwise approach with 1.404 mL every 5 min ($0.281 \text{ mL/min} \times 5 \text{ min}$) for the first 20 min and 2.809 mL every 10 min thereafter, decreasing the pH from 6.0 ± 0.5 to $\text{pH } 2.0 \pm 0.5$. The RGE solution (or water for the no enzyme control experiments) was added at 0.0740 mL every 5 min for the first 20 min and 0.148 mL every 10 min thereafter. Gastric contents were mixed manually right after the additions of digestive fluids. To standardise stirring, the same person carried out stirring with the plastic pipette used to add the solution using 5 rotations each time. In accordance with the recommendation of Mulet-Cabero, Egger, et al. (2020), the duration of the gastric phase was calculated as twice the half-gastric emptying time, as estimated based on the caloric content of the meal to be digested *in vivo* and the assumption that the gastric content will be emptied at a mean rate of 2 kcal/min from ingestion to the half-gastric emptying time. This is intended to digest the milk samples in the same conditions to which they would be exposed when consumed as part of a meal *in vivo*. Our present study mimics the *in vivo* digestion of a 200 mL cup of whole milk containing 0.77 kcal/mL, leading to a total caloric content of 15.4 kcal for our meal. This corresponds to a half-gastric emptying time of 38.5 min, that was rounded up to 40 min to allow gastric emptying every 10 min for simplification. We also added 10 min at the end of our digestion to allow for a fat measurement sequence to be run on the MRI, bringing our total time of digestion to 90 min. Gastric emptying was carried out from the bottom of the vessel every 10 min up to 90 min using a 10 mL plastic pipette with a cut tip (inserted into digesta from above) to allow for particles <3 mm to be collected with the fluid (total of 4.5 mL each time). These samples were then stabilised by heating in a thermal block set to 100 °C for 10 min in order to reach a $T^\circ\text{C} > 80^\circ\text{C}$ for 5 min (measured inside the Eppendorf), before cooling on ice. The pH of the emptied samples was measured approximately 10 min later to monitor the kinetics of gastric acidification, and stored at -20°C until required for analysis.

2.4.3. Temperature control

The temperature within the gastric digestion vessel was controlled using a circulating cryobath. To mimic the increase/decrease of gastric temperature seen in the *in vivo* situation, including the pre-heating or cooling in the mouth and oesophageal tube, different temperature kinetics were designed. When 350–400 mL of coffee or orange juice are consumed at 4 °C, the gastric temperature reaches about 22 °C right

after ingestion and returns to body temperature (37 °C) within 20–30 min (McArthur & Feldman, 1989; Sun et al., 1988). Likewise, the consumption of hot versions of these liquid foods lead to an initial gastric temperature of approximately 44 °C, returning to body temperature 20–30 min after consumption (McArthur & Feldman, 1989; Sun et al., 1988). This is why milk was added to the gastric digestion vessel at 22 °C to replicate the consumption of milk at 4 °C and at 44 °C to replicate the consumption of milk at 60 °C. To ensure these temperature kinetics, different cryobath settings were developed for each consumption temperature. For the 4 °C consumption temperature, the cryobath was initially set to 22 °C, and ramped to reach 37 °C over 30 min inside the vessel. For the 60 °C consumption temperature, the vessel was initially heated to 44 °C and subsequently cooled to reach 37 °C over 30 min. In the control experiment, in which the consumption of milk at physiological temperature (37 °C) was simulated, gastric digestion was performed at a constant temperature. Temperature inside the vessel was continuously monitored using a pre-calibrated optical fibre connected to a data logger (UMI8, FISO Technologies Inc., Canada).

2.5. MRI acquisition

MRI experiments were carried out on a 1.5T MRI device (Magnetom, Avanto, Siemens, Germany) with a 4-element head RF receiver coil. The following protocols for the acquisition of tri-dimensional (3D) images were used:

A fast recovery turbo spin echo (FR-TSE) sequence for morphological images with effective echo time equal to echo spacing 9.8 ms, Turbo Factor 6, TR 150 ms, FOV 86 mm × 86 mm × 43 mm, matrix size $128 \times 128 \times 64$, bandwidth 200 Hz/pixel, 1 scan. Acquisition time was 3 min 51 s and image resolution was $0.7 \text{ mm} \times 0.7 \text{ mm} \times 0.7 \text{ mm}$.

A gradient multi-echo (GRE) sequence to separate water and lipids signals as described in Hernando, Kellman, Haldar, and Liang (2010). Parameters were set at first TE 1.48 ms, echo spacing 2.81 ms, 6 echoes per echo train, TR 75 ms, flip angle 15°, FOV $112 \text{ mm} \times 128 \text{ mm} \times 64 \text{ mm}$, matrix size $112 \times 128 \times 64$, bandwidth 888 Hz/pixel, 1 scan. Acquisition time was 8 min 59 s and image resolution was $1 \text{ mm} \times 1 \text{ mm} \times 1 \text{ mm}$.

FR-TSE sequence was carried out every 5 min between $t = 0$ and 40 min, and between $t = 50$ and 75 min. A final FR-TSE sequence was carried out at $t = 85$ min. A GRE sequence was carried out between $t = 40$ and 50 min and between $t = 75$ and 85 min. To carry out a GRE sequence, the water flow to heat the vessel was halted to prevent artefacts from appearing in the images.

2.6. Estimation of the particle volume, number and size from MRI

The volume of digesta was semi-manually analysed in FR-TSE images using Avizo 3D Pro software (FEI Company, version 2021.2). Thresholding was carried out to segment digesta from the digestion vessel and external environment. The threshold value was selected by visual inspection. This value was adjusted for each 3D image, as the contrast between the digesta voxels and the background was not constant. The fill function was used to fill any gaps in the digesta that were not automatically detected. In some cases, such as when the optic fibre was visible in the images, manual editing was carried out. The selected voxels were then used to compute a total digesta volume. Within digesta volumes, particles were characterized using Label Analysis Avizo function. This function was applied on binary images, where particles were manually thresholded. The algorithm first identified particles by applying a label to connected voxels (3D neighbours segmented voxels), so as each particle was assigned to a unique label. Then the total particle volume and the number of individual particles were quantified. To estimate the average particle size, the total particle volume was divided by the number of particles. This was carried out for all 3 repetitions at each condition.

2.7. Lipid quantification

Separation of water and lipid signals was carried out from the 3D GRE images using the “water-fat separation” method detailed in [Picaud, Collewet, and Idier \(2016\)](#). This method is based on the difference in signal frequency between the hydrogen protons of water molecules and those of lipid molecules. It enables the disentanglement of the water signal from that of lipids in each voxel. The method requires the composition of the lipids since the frequency of the lipid protons signal depends on their position on the triglyceride chain as described in [Berglund, Ahlström, and Kullberg \(2012\)](#). For the composition of triglycerides, we used one composition of dairy fat data provided in <http://www.agroscope.admin.ch/agroscope/fr/home/themes/denrees-alimentaires/alimentation-sante/lait-produits-laitiers/graisse-du-lait.html>, and which is reproduced in [Supplementary Material S1](#). Using this data, we calculated the average number of protons at each position on the triglyceride chain and established one average frequency spectrum of the lipids described by nine different frequencies.

For each pixel of the 3D images, the signal of the lipids was quantified and then converted to grams by using a dedicated experiment on the rehydrated full-fat milk alone. 2D vertical lipid maps were finally obtained by averaging the lipid maps over one of the horizontal direction regarding the vessel. The reduction of information from 3D to 2D was necessary in order to improve the signal-to-noise ratio of the result and also to facilitate interpretation. However, 3D acquisitions were relevant since they made it possible to measure the lipids in the whole vessel.

2.8. Analysis of digestion samples

All samples were centrifuged (3000 g, 5 min, 4 °C) and the supernatant was further filtered through 0.8 µm syringe filters (Acrodisc® 25 mm w/0.8 µm Supor® STRL, Pall Corporation) to remove particles and dairy fat. In the no-enzyme control experiments, the samples collected at $t = 10$ and 20 min of digestion were still liquid. As those liquid samples were useless to monitor the release of proteins/peptides from the particles, they were not analysed.

2.8.1. Released proteins

The concentration of soluble proteins, released peptides and amino acids in the supernatant, further referred to as released proteins, was measured using 4th derivative UV absorbance using the method proposed by [Lüthi-Peng & Puhan, 1999](#) that is insensitive to the whey proteins to caseins ratio. This was performed using a 6M guanidine-HCl and 0.1M Na acetate buffer at pH 5.0, quartz cuvettes, and a UV spectrophotometer (UVmc2, Safas Monaco, Monaco, France). Results were converted into masses using a calibration curve established with skim milk as a standard (0–1.5 g/L of proteins) and the volume of the digesta at the time at which the samples were collected. The mass of released proteins that was withdrawn during preceding gastric emptying steps was then added to the data obtained at time t to compute the masses (mg) of released proteins into the digesta supernatant.

2.8.2. Free primary amines (NH_2)

The release of free primary amino groups (NH_2) in the supernatant, which is indicative of both the mass and the length of released peptides, was measured using a previously described o-phthalaldehyde (OPA) spectrophotometric assay in microplates [\(Lorieau et al., 2018\)](#) that was adapted from the protocol of [\(Church, Swaisgood, Porter, & Catignani, 1983\)](#). Results were corrected for digestive secretions (blanks with no food), and converted into free primary NH_2 quantities (mmol) using a calibration curve created with L-methionine (0–2 mM) and the volume of the digesta at the time at which the sample were collected. The NH_2 quantity that had been withdrawn during preceding gastric emptying steps was then added to the data obtained at time t in order to compute the quantity of free primary NH_2 (mmol) in the digesta supernatant.

2.9. Statistical analysis

Results are presented as mean \pm standard deviation of three replicates unless otherwise stated. The authors acknowledge that the standard deviation may be underestimated due to low number of replicates ($n = 3$) [\(Synek, 2008\)](#). Statistical significance between experimental conditions was assessed at each time point using a two-way analysis of variance (ANOVA) and post hoc pairwise comparisons using the Tukey's HSD test. This analysis was performed with GraphPad Prism software (Prism for Windows, Version 9.0.1) and a threshold for significance of $p = 0.05$.

3. Results

3.1. Gastric temperature

The temperature curves obtained in digesta under MRI experiments are presented in [Fig. 1](#). As described previously, these curves were carefully designed to match as closely as possible to *in vivo* data available for liquid foods in the literature. Those temperature profiles are similar to the ones used by [Yang et al. \(2023\)](#) who studied the effect of consumption temperature of skim milk on its *in vitro* gastric digestion, but with further consideration of the temperature change that occurs in liquid foods before reaching the human stomach [\(McArthur & Feldman, 1989; Sun et al., 1988\)](#). This is why, for instance, the gastric digestion of the cold (4 °C) milk began at 22 °C in our experiments and not 4 °C as in [Yang et al. \(2023\)](#). In the same way, in the experiment replicating the consumption of hot (60 °C) milk, gastric digestion was initiated at 44 °C. During gastric digestion of the 4 °C and 60 °C milk samples, the standard digestion temperature (37 °C) was reached, on average, after 30 min. Note that the drops in temperature curves beyond 50 min were due to switching off water circulation to allow images for lipid quantification to be acquired.

3.2. Gastric pH

The mean pH profile of each experimental condition is shown in [Fig. 2](#). The pH increased rapidly from 2 (basal secretions) up to approximately 6 upon the addition of the milk. Following this, the pH decreased continuously throughout digestion reaching values around 4 halfway through the digestion and pH 2 by the end of the experiments, as recommended in the semi-dynamic INFOGEST protocol [\(Mulet-Cabero, Egger, et al., 2020\)](#). All curves followed a similar trend except for the no enzyme control, for which the pH tended to decrease faster below

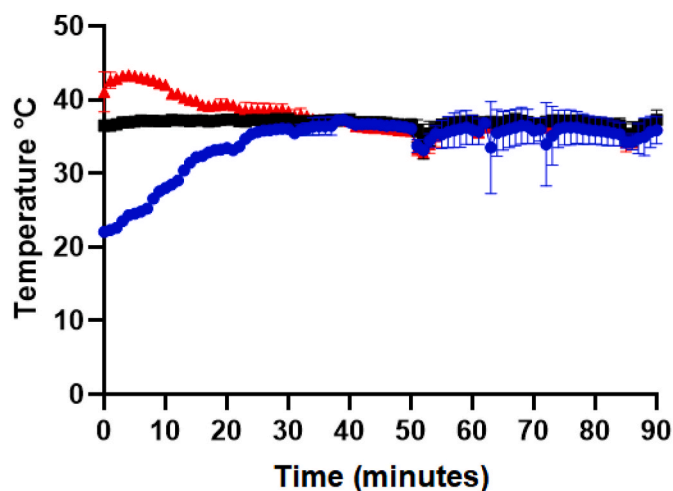


Fig. 1. Temperature during gastric digestion of milk: at 4 °C (●), at 37 °C (■), and at 60 °C (▲). Data are means \pm SD over three replicates.

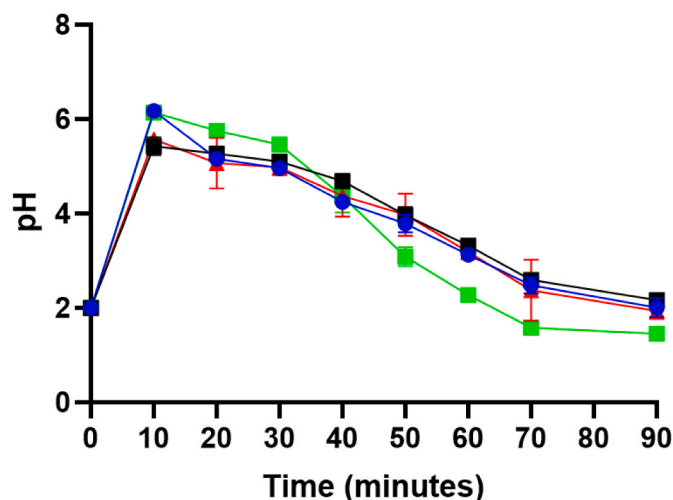


Fig. 2. pH during gastric digestion of milk: at 4 °C (●), at 37 °C in the absence (■) and in the presence of gastric enzymes (■), and at 60 °C (▲). Data are means \pm SD over 3 replicates.

pH 4. This is due to the lack of enzymatic hydrolysis of proteins in this experiment. When peptide bonds are hydrolysed at acidic pH by pepsin, some protons are consumed by the amino groups ($-\text{NH}_2$) at the *N*-terminus of the peptides produced. This pepsin-related consumption of protons, which adds to the initial buffering capacity of the food, does not exist in the absence of enzymes and explains why the pH decreases faster. This is a well-known phenomenon that can even be used to monitor pepsin activity with a pH-stat device (Mat, Cattenoz, Souchon, Michon, & Le Feunteun, 2018).

3.3. MRI monitoring of particle formation and disintegration

Fig. 3 presents the middle slice of FR-TSE MRI 3D images acquired during the first 32.5 min of digestion and at the end of the experiments. In these images, the contrast is mainly due to differences in T2 relaxation time, which may be interpreted as a probe of the biopolymer structure and concentration (Mariette, 2009). Because no significant change in protein structure and water content occur during gel formation, a similar MRI dark grey signal was observed before and after milk coagulation. It was, therefore, not possible to distinguish liquid milk from a macroscopic gel spanning over the entire vessel in these images (Fig. 3).

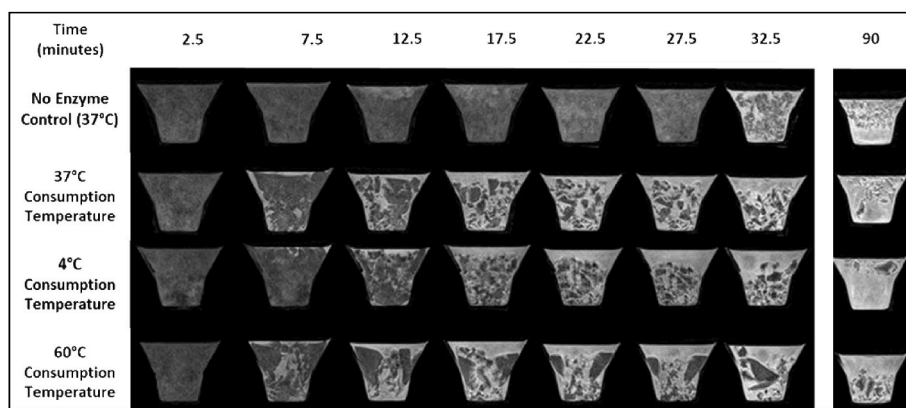


Fig. 3. Examples of MRI images (from 1 replicate over the 3) acquired with the Fast Recovery Turbo Spin Echo (FR-TSE) sequence, illustrating the monitoring of gel formation and subsequent disintegration of dairy gel particles. Prior to onset of coagulation, the digesta, which is liquid, appears dark. After onset of coagulation, liquid digesta appears bright, and coagulated particles appear dark. Each time point involved the acquisition of 3D data sets comprising 64 images, capturing the entirety of the digestion vessel. The presented images showcase the middle slice from each acquisition, specifically focusing on those obtained during the first 32.5 min of digestion and at the conclusion of the experiments. For a comprehensive overview of the entire kinetics in each condition, refer to the [Supplementary Material S2](#).

Consequently, gel particles, which appear dark within a bright surrounding solution, were only visible in the next MRI image, i.e. after the gel was broken down into pieces by the manual mixing step between MRI scans: at $t = 32.5$ min with the no-enzymes control at 37 °C, from $t = 12.5$ min with the milk at 4 °C, and at $t = 7.5$ min with the milks at 37 °C and 60 °C. This was true for all 3 replicates for each condition (Supplementary Material S2). Knowing that those times correspond to the mean time of the FR-TSE MRI 3D acquisitions, and that these lasted for 5 min, it may be concluded that milk coagulation took place within 25–30 min in the absence of enzymes, within 5–10 min for the milk at 4 °C, and within 0–5 min for the milks at 37 °C and 60 °C. These ranges of coagulation times were supported by our visual observations made when fresh gastric secretions were added to the milk between successive MRI scans. They were later confirmed by trying to study the effect of the initial gastric temperature (22 °C, 37 °C and 44 °C) on the onset of milk coagulation induced by the basal secretions (no further SGF addition) using rheological measurements. A coagulation time of 27.7 ± 4.2 min (triplicates) at a constant temperature of 22 °C was found, while coagulation was so quick at 37 °C and 44 °C (≤ 30 s) that it was not possible to obtain fair and repeatable rheological measurements (Data not shown).

The FR-TSE images shown in Fig. 3 were further processed to characterize the particles observed throughout gastric digestion in terms of total volume, number, and mean volume per particle. These results are presented in Fig. 4, in which the data from the control experiments with and without enzymes at 37 °C are presented on the left-hand side (Fig. 4A, C and 4E), and the data obtained at the three studied consumption temperatures in the presence of enzymes are presented on the right-hand side (Fig. 4B, D, 4F).

At 37 °C in the presence of enzymes, the particle total volume (Fig. 4A) and the mean volume per particle (Fig. 4E) were maximal right after the gel formation, that is at $t = 2.5$ min. Just after the breaking down of the gel into particles upon stirring, at $t = 7.5$ min, a reduction of the total particle volume by half in only ~ 5 min was observed (Fig. 4A). Because the pH was still unfavourable for pepsin action at that time (Fig. 2), and because gastric emptying was not undertaken between $t = 2.5$ and 7.5 min, this result can only be explained by an intense syneresis of the particles, i.e. particle shrinking with whey expulsion. Later on, the decrease in particle total volume was more gradual. Conversely, in the absence of enzymes, the mean and total particle volumes remained negligible until the onset of coagulation (between 25 and 30 min) and was maximal around 35 min, remaining stable until the end of the experiments (Fig. 4A and E). Although caution must be taken with these results of semi-automatized analyses, large numbers of very small

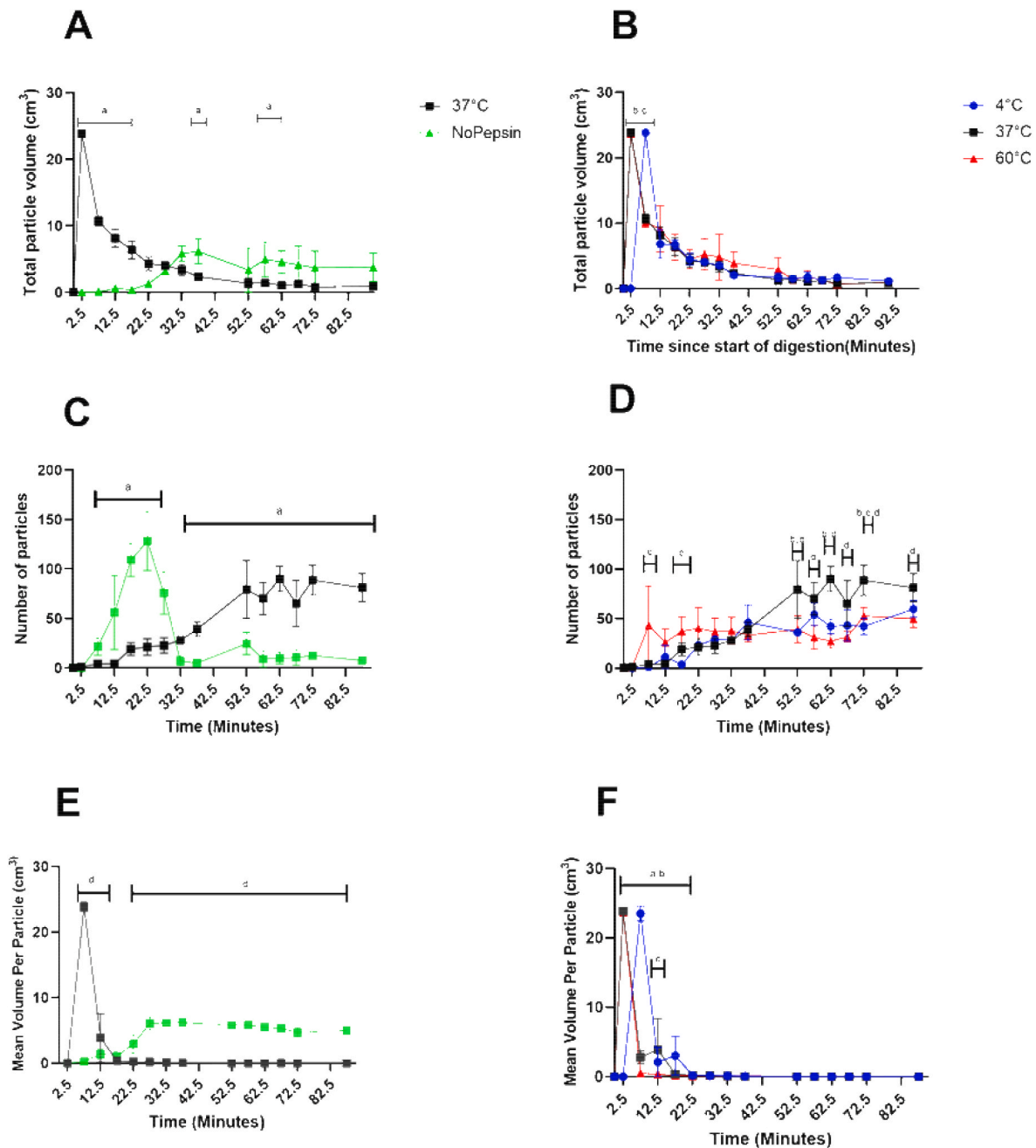


Fig. 4. Particle analysis of MRI images. The left-hand-side subplots (A, C, E) compare the results obtained at 37°C in the presence (■) and absence of enzymes (▲). The right-hand-side subplots (B, D, F), compare the results obtained in the presence of enzymes at 4 °C (●), 37°C (■) and 60°C (▲). Data are means \pm SD over 3 replicates. Statistical differences ($p < 0.05$) are indicated by letters (a: 37 °C with enzymes vs 37°C without enzymes; b: 4 °C vs 37 °C, c: 4 °C vs 60 °C, d: 37 °C vs 60 °C.).

particles (Fig. 4C) seemed to form in the no pepsin control before coagulation was initiated, peaking at 22.5 min (pH \sim 5, Figs. 2, 4C and 4E), a phenomenon that could be attributed to the local formation of particles when adding the drops of HCl. When coming closer to the isoelectric point of caseins, a sharp decrease in particle number and an increase in the total and mean particle volumes occurred, indicating that larger particles were being formed. During the second half of gastric digestion, when pH conditions were conducive to casein aggregation, but also most favourable to pepsin action, the total and mean particle volumes remained higher in the no enzyme control than in the digestions containing pepsin. The opposite was observed with the number of particles, which was higher for the enzyme experiment confirming that pepsin was the main causative factor in the breakdown of particles.

Turning to the effect of milk temperature consumption (Fig. 4B, D and 4F), some significant differences in particle total volume (Fig. 4B), number of particles (Fig. 4D) as well as mean volume per particle (Fig. 4F) were observed for the milk consumed at 4 °C compared to either 37 °C or 60 °C for $t \leq 12.5$ min ($p < 0.05$) because of its delayed coagulation. Of note is that at $t = 7.5$ min, the particle total volumes were 10.9 ± 5.5 cm³ at 37 °C and 10.1 ± 6.3 cm³ at 60 °C, corresponding to 41.3% and 40.4% of total digesta volume, respectively. These values further show that the gel particles underwent a significant syneresis after the breaking down of these gels, and that this phenomenon was of similar magnitude for both the warm and hot milks. Interestingly, after the breaking down of the gel into particles with the milk at 4 °C, from $t = 12.5$ min (Fig. 4B), initial differences vanished and

the total particle volume became very similar for all the studied temperatures at all time points (Fig. 4B), with no statistical differences in terms of mean volume per particle (Fig. 4F). Despite different milk coagulation kinetics and properties in the early stages of gastric digestion, the overall behaviours of the particles were thus comparable later on, consistent with pepsin action being the main factor in the breakdown of particles, even though the number of particles in the 37 °C experiments tended to be slightly higher than for the 4 °C and 60 °C milks from $t = 57.5$ min.

3.4. Fat quantification by MRI

Fig. 5A shows the spatial distribution of lipids at the mid-point ($t = 40$ min) and at the end ($t = 80$ min) of gastric *in vitro* digestions in the presence of enzymes Fig. 5B shows the estimated lipid mass as a function of the height in the vessel. At $t = 40$ min, when milk is consumed at 4 °C, lipids were found to be spread between the bottom and top of the digestion vessel, while when consumed at 37 °C and 60 °C, lipids remained at the bottom of the vessel with no lipid creaming to the top. At $t = 80$ min (end of digestion), all the lipids had migrated to the top of the vessel for the milk consumed at 4 °C, most lipids had creamed when milk was consumed at 37 °C, and all lipids remained at the bottom when milk was consumed at 60 °C. The same observation could be made for the particles in the FR-TSE images (Fig. 3 and Supplementary Material S2), in which the particles tended to float to the surface around mid-digestion with the milk at 4 °C, at the end of the digestions with the milk at 37 °C, but never with the milk at 60 °C. This indicates that the lipids observed in Fig. 5 are not free lipids alone, they rather correspond to milk fat entrapped within the particles.

3.5. Protein analysis in the digesta supernatant

Fig. 6A and B depict the mass of proteins/peptides released into the supernatant, and Fig. 6C and D shows the quantity of free primary amines in the digesta supernatant. Of note is that only the samples collected after the onset of gel formation were considered in the no enzyme control experiment, that is for $t \geq 30$ min. As expected, both the mass of proteins and quantity of free amines released into the supernatant at 37 °C were significantly higher in the presence of enzymes than in their absence ($p < 0.0001$, Fig. 6A and C from $t = 30$ –40 min). In the absence of enzymes, the estimated mass of proteins/peptides released into the supernatant plateaued at around 120 mg (Fig. 6A). Considering that the amount of proteins in the milk was initially close to 800 mg, it can be estimated that about 15% of the initial milk protein content was released into the supernatant by the end of gastric digestion. This value, which is close to the expected 20% of whey proteins in the milk, is thus compatible with a release of whey proteins from the particles into the supernatant. In the presence of enzymes at 37 °C, coagulation occurred by the first emptying point, hence leading to a gradual release of proteins into the supernatant that is indicative of the breakdown of protein particles into peptides that release into the supernatant. The rise in the quantity of free amines and mass of protein in the digesta within the first 30 min, when the pH was above 5 (Fig. 2), indicates that protein hydrolysis by pepsin was effective from the early stages of gastric digestion. The differences in the free amines released in the presence and absence of enzymes at 37 °C were significant from $t = 30$ min. The masses of protein released under both conditions were similar at this stage, but the curves started diverging from this point, and the differences became significant from $t = 40$ min. During the second half of digestions (after $t = 40$ min), the magnitude of the gap between the results obtained with and without enzymes increased rapidly, hence suggesting that pepsin activity increased significantly when the pH was brought down to 5 and below (Fig. 2). According to the results of both Fig. 6B and D, the release kinetics of soluble peptides did not depend on the milk consumption temperature, however. In the presence of enzymes, the estimated mass of proteins/peptides released into the

supernatant was about 350–400 mg in all experiments (Fig. 6B), indicating that about half of the initial protein milk content was emptied from the gastric digesta in the form of soluble proteins and peptides during the course of the experiments. The other half was therefore emptied from the gastric digesta in the form of dairy particles.

4. Discussion

Thanks to the use of MRI and gastric temperature profiles relevant to the digestion of cold (4 °C), warm (37 °C) and hot (60 °C) liquids in humans, the present study shows that the consumption temperature can influence the behaviour of full-fat milk during semi-dynamic gastric *in vitro* digestion. The delay before the onset of milk coagulation and the creaming properties of the fat-rich dairy particles appeared to be particularly affected by the milk temperature. However, both the kinetics of particle disintegration monitored by MRI, and the release kinetics of peptides into the supernatant remained largely unaffected.

4.1. Particle formation and disintegration

In the absence of digestive enzymes at 37 °C, the formation of milk particles occurred due to the increasing acidity, when pH approached the isoelectric point of caseins. This phenomenon took place within 25–30 min, which coincided with a pH of ~ 5 (Fig. 2). This result is in excellent agreement with the knowledge on acid-induced milk coagulation, showing that the onset of milk coagulation typically occurs around that pH (Lucey & Singh, 1997). Past this time point, casein particles were progressively diluted due to further additions of the acidic secretions and gastric emptying steps but no breakdown of particles (Fig. 4E) nor any gradual release of peptides (Fig. 6A and C) was seen in the absence of pepsin. These observations show that the strong acidity of the gastric environment was not sufficient by itself to disintegrate the dairy particles. In the presence of enzymes, the onset of milk coagulation took place much more rapidly for all milk consumption temperatures; that is in less than 10 min, at $\text{pH} \geq 5.5$, and before the first addition of SGF on top of the basal secretions. These findings are in excellent agreement with the study of Mulet-Cabero, Mackie, Wilde, Fenelon, and Brodkorb (2019) on the semi-dynamic *in vitro* gastric digestion of different full-fat milks, which all coagulated in less than 10 min as well. They also confirm the key role of pepsin in the formation of the milk coagulum during gastric digestion, and indicate that enough pepsin was already contained in the basal secretions to reach the critical amount of κ -casein hydrolysis for coagulation to occur at all considered temperatures and despite a pH still close to 6. Although pepsin is often considered to be active at low pH only, this is not the case when it is acting on casein micelles. It has indeed long been known that pepsin can be active on caseins at pH close to neutrality, as recently reemphasised by Salelles et al. (2021) who have investigated the dependence of pepsin activity as a function of pH on casein microparticles. According to another recent study, approximately 73% and 33% of κ -caseins must be hydrolysed at pH 6.3 and 5.3 to induce coagulation, respectively, hence suggesting that about half of the κ -caseins contained in our milk could be hydrolysed within the first minutes of gastric digestion (Yang et al., 2022).

Our results further show an increased time to protein coagulation, by 5–10 min in our experimental conditions, when milk was consumed at 4 °C compared to 37 °C or 60 °C (Figs. 3 and 4B). This observation was reinforced by our rheological trials in the presence of basal gastric secretions showing that milk coagulation occurred in more than 20 min at 22 °C but in less than 1 min at 37 °C and 60 °C. These data are consistent with the study of Yang et al. (2023), who also reported a delayed coagulation of skim milk with a decreasing consumption temperature. Although the literature lacks data on the effect of temperature at high pH on rabbit pepsin acting on caseins, as used in the present study, it is well known that the activity of pepsin increases with temperature. It has for instance been reported that porcine pepsin activity on haemoglobin at pH 2 can be about twice as high at 44 °C than at 22 °C (Brier et al.,

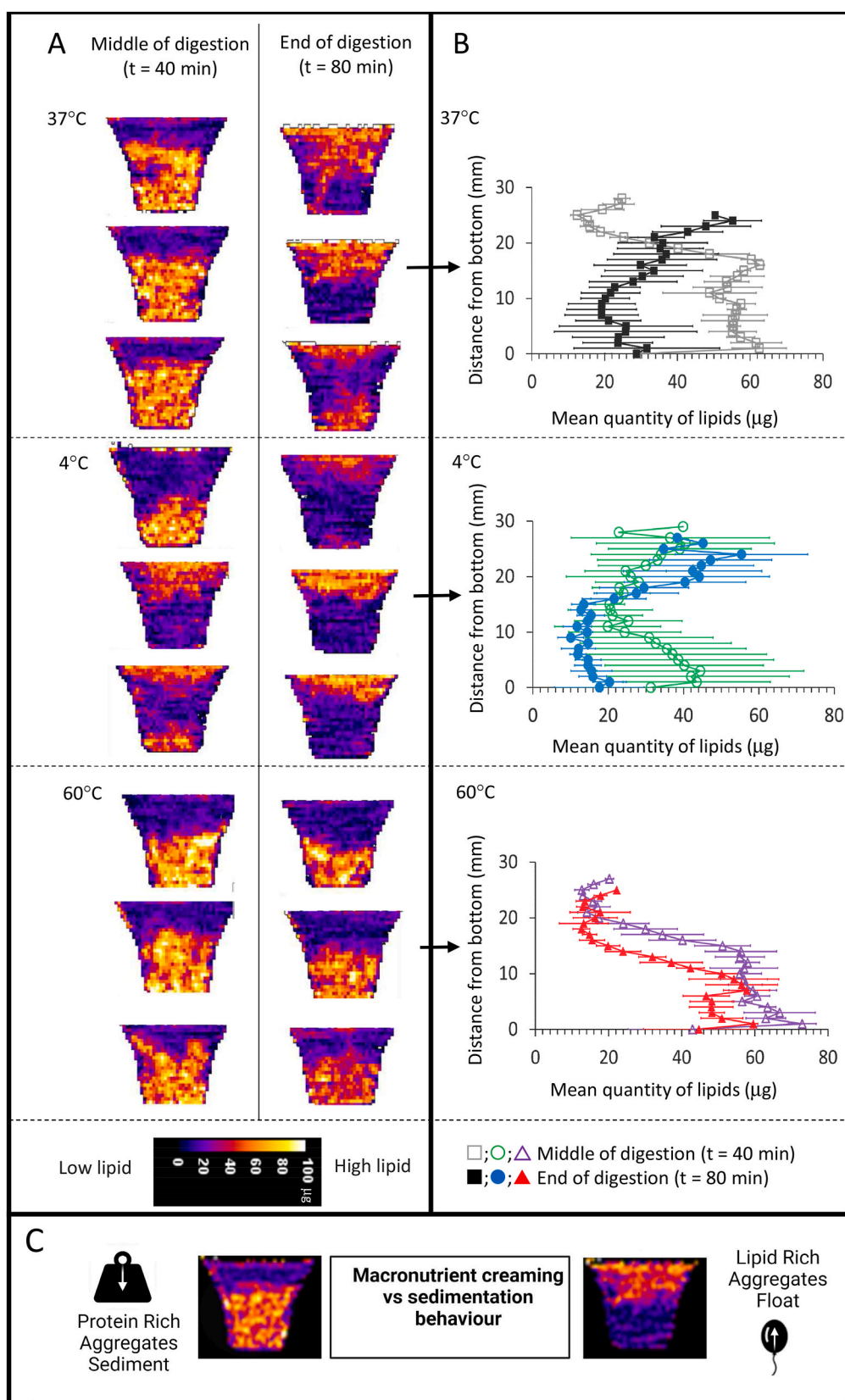


Fig. 5. (A) Colour images represent lipid maps (mean value over the projection direction) at t = 40 (Left Column) and t = 80 min (Right Column) for the three replicates. Orange colour represents areas of high lipid amount. (B) Graphs represent the mean quantity of lipid signal over 3 repetitions calculated at each millimetre along the height of the digestion vessel. (C) Schematic representation of the effect of proteins vs lipids in particles in relation to creaming and sedimentation. Lipids within particles act like balloons, causing particles to cream to the top, while proteins act like anchors, causing particles to sediment to the bottom.

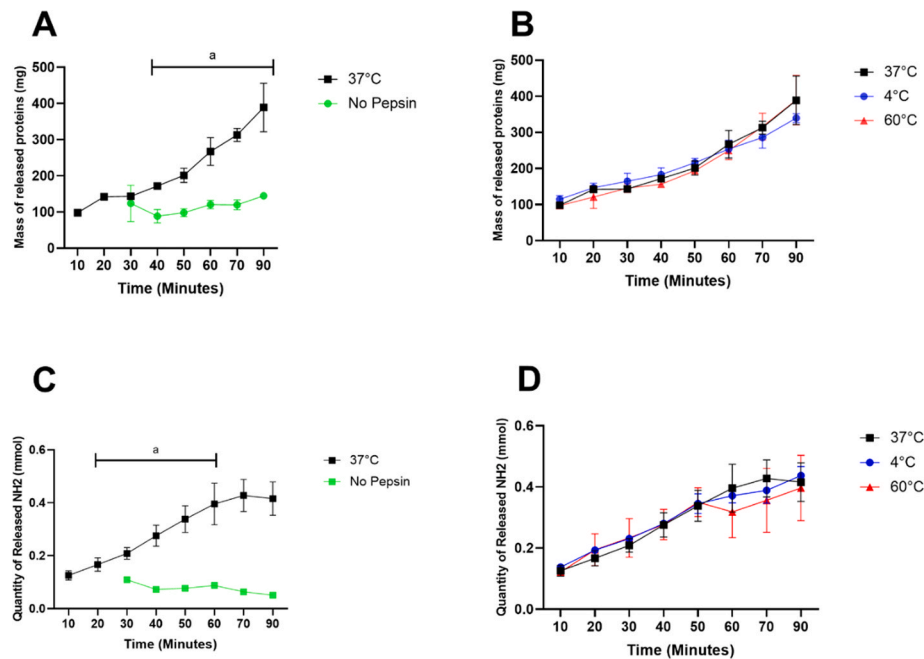


Fig. 6. Release of proteins/peptides and free primary amines in the digesta supernatant. The left-hand-side subplots compare the mass of released peptides (A) and the quantity of released free NH₂ (C) obtained in the presence (■) and absence (●) of enzymes (■). The right-hand-side subplots compare the mass of released peptides (B) and the quantity of released free NH₂ (D) obtained in the presence of enzymes at 4 °C (●), 37 °C (■) and 60 °C (▲). Statistical differences ($p < 0.0001$) are indicated by letters (a: 37 °C with enzymes vs 37 °C without enzymes).

2007). The high sensitivity of pepsin to temperature therefore explains why the critical level of κ -casein hydrolysis needed to induce the formation of dairy particles was reached later with the lukewarm temperature (~ 22 °C) used at the start of the digestion of the 4 °C milk.

However, no significant variation could be found in terms of protein hydrolysis during gastric digestion as a function of the milk consumption temperature (Fig. 6B). The same was true for the evolution of the total volume of particles estimated from the FR-TSE MRI images (Fig. 4B). These findings suggest that the progressive breakdown of particles upon pepsin action was largely independent of milk consumption temperature in our experimental conditions. This contrasts somehow with the results obtained by Yang et al. (2023) on the effect of the consumption temperature of skim milk on gastric digestion. In their study, protein hydrolysis was found to be relatively faster for a 4 °C milk than for milks at 37 °C and 50 °C, a phenomenon that was attributed to the looser and softer protein network structure formed with the 4 °C milk during the early stages of digestion. They also reported that, after 240 min of digestion, the total curd weights of the warm and hot skim milks were higher than that of the 4 °C milk. The discrepancy between these findings and ours might relate to the presence of lipids in full fat milk, either in terms of interfacial dynamics and/or lipid crystals for the period below 37 °C, that may have increased the firmness of the curds formed during our experiments. The experimental conditions used in both studies most certainly influenced the results as well. In the study of Yang et al. (2023), the starting gastric temperatures were initially set at 4 °C and 50 °C to investigate the digestion of cold and hot milk, with a return to 37 °C within 60 min. In the present study, our analysis of the *in vivo* literature led us to consider starting temperatures of 22 °C and 44 °C with a return to 37 °C within about 30 min as realistic physiological conditions for the same purposes. This is because the temperature of a drink changes significantly before it reaches the stomach according to the human studies we could find (McArthur & Feldman, 1989; Sun et al., 1988). Moreover, while we used a stepwise mixing procedure to allow for MRI acquisitions to be performed in the absence of fluid movements, Yang et al. (2023) used the Human Gastric Simulator (HGS), which enables a more reliable simulation of the mixing that should occur within the stomach. In comparison to our protocol parameters, the

mixing conditions and the temperature profiles used in the study of Yang et al. (2023) should both contribute to the formation of more fragile dairy particles with the milk at 4 °C. All these considerations are likely to explain why they observed a small but significant increase in protein hydrolysis with cold milk, while we did not. More studies with considerations of physiologically relevant gastric mixing, gastric temperature profile, and gastric emptying (which also seemed different in both studies) are thus needed to better evaluate if the consumption of cold milk can significantly influence the extent of protein hydrolysis at the gastric stage. In any case, the combined results of both studies suggest that such an effect should remain of a rather limited extent. Employing more advanced analytical techniques such as amino acid analysis, as in Jacobs et al. (2019), may also appear as an interesting mean to obtain more information on the breakdown of the dairy particles and to estimate the caseins to whey proteins ratio in the emptied material and/or the released peptides.

4.2. Creaming of fatty particles

Another phenomenon we observed is that the dairy particles tended to cream during gastric digestion (Figs. 3 and 5). To understand this phenomenon, one needs to understand why the density of these fat-rich particles can decrease during digestion. As schematically represented in Fig. 5C, an analogy can be used with lipids acting as balloons, causing particles to rise to the top, and with proteins acting as anchors, causing particles to sediment to the bottom. With this analogy, the creaming of the particles can be ascribed to a progressive increase of the lipid-to-protein ratio within the particles during digestion, or in other words, the release of proteins within the supernatant was more rapid than for lipids. This could happen because of a tendency of the lipid droplets to remain attached and accumulate at the periphery of the eroding protein network, a phenomenon that has been observed using confocal microscopy (Luo, Ye, Wolber, & Singh, 2021). This could also be explained by a progressive release of proteins that were not directly involved within the protein network. This second mechanism is likely to have occurred during our experiments since some dairy proteins, notably whey proteins, can remain solubilized or suspended within the aqueous phase

upon milk coagulation and thus be progressively expelled from the particles later on. Interestingly, such a process should be enhanced as a function of digestion time because of the increasing dilution of the digesta by the gastric secretions. This could indeed conveniently explain why the particles also creamed before the end of the digestion experiments in the absence of digestive enzymes at 37 °C (Fig. 4).

Moreover, the time at which the dairy particles creamed was also influenced by the milk consumption temperature (Fig. 5). When milk was consumed at 4 °C, the lipid-rich particles creamed in the middle of digestion. The same phenomenon took place later with the milk at 37 °C, between $t = 40$ and 80 min, while the lipid-rich particles always remained sediment at the bottom of the vessel when milk was consumed at 60 °C. Several hypotheses could be put forward to explain this effect of consumption temperature on the kinetics of particle creaming. For instance, the amount of β -caseins in casein micelles has been shown to be temperature-dependent, with up to 60% of β -caseins that can be removed from milk when centrifuged at 4 °C, though noting that the milk temperature was only brought down to 22 °C to simulate the behaviour of a 4 °C milk (McMahon & Oommen, 2008). It could nonetheless be assumed that there was a lower content of β -caseins within the dairy particles formed with the cold milk, or a more rapid protein impoverishment of the particles, leading to a sooner creaming than for the hot milk. A similar reasoning could be put forward based on the assumption that a looser curd was formed when milk was consumed at 4 °C when compared to 60 °C, consistent with the findings of Yang et al. (2023). Additionally, it is also possible that a firmer curd formed at high temperatures would be more resistant to pepsin action, even though we fail to see significant differences during our monitoring of the disintegration of dairy particles and the release of peptides. It is also noteworthy that milk fats are partially in their solid form below 37 °C and that crystalline milk fat is less susceptible to lipolysis (Smoczyński, 2017). It is difficult to know whether these phenomena could also contribute to the earlier creaming of dairy particles at low consumption temperature. Considering that the minimum temperature used in the gastric phase was 22 °C, and that the milk fat should have been in a comparable state with the milks at 37 °C and 60 °C, any interpretation of our results based on the lipid melting point is quite unlikely. More studies would actually be welcome to better understand the exact mechanisms explaining how the consumption temperature of whole milk may influence the kinetics of particle creaming.

4.3. Physiological relevance

The observed differences in both the kinetics of protein coagulation and particle creaming may affect the rate of nutrient delivery to the small intestine *in vivo*. This is because the gastric behaviour of dairy products can directly influence the composition of the material that is emptied by the stomach. For instance, Mulet-Cabero, Rigby, Brodtkorb, and Mackie (2017) demonstrated *in vitro* that, when digesting a liquid oil-in-water emulsion vs. a semi-solid dairy product made of yogurt and Gouda cheese particles, the emptying of lipids was delayed with the liquid emulsion due to lipid creaming, leading to a low amount of lipids available for intestinal absorption during the early stages of digestion. A similar phenomenon may therefore also apply when particles containing lipids tend to float during gastric digestion, as observed with our 4 °C and 37 °C milks. In Mulet-Cabero et al. (2017), the lipids contained in the semi-solid dairy product were entrapped within the food matrix and tended to remain sediment to the bottom of the digestion vessel, similar to what we observed for the milk at 60 °C. Although liquid milk was added to the digestion vessel in the present study, the very rapid formation of dairy particles entrapping lipids at high temperatures resulted in a system very similar to the semi-solid matrix investigated in the study of Mulet-Cabero et al. (2017). Similar kinds of phenomena have also been observed *in vivo* with pigs fed with differently processed milks (Ahlborn, et al., 2023). In that study, it was shown that the fat from non-homogenised milks tended to cream and induce a much slower

release of lipids to the small intestine (lipid half gastric emptying time = 93 min and 228 min) than for homogenised milks that are less prone to fat creaming (lipid half gastric emptying time = 11 and 51 min). Therefore, one potential physiologically relevant outcome of the present study is that lipids may be emptied more rapidly into the small intestine when milk is consumed hot compared to cold or body temperature. Interestingly, a human study by Mackie, Rafiee, Malcolm, Salt, and van Aken (2013) also suggests that the slower emptying of semi-solid dairy food as compared to liquid foods can be accompanied by enhanced appetite suppression. This increased appetite suppression was concluded to be due to the retention of food boluses in the antrum of the stomach activating mechanoreceptors within the gastric wall (Mackie et al., 2013). It is therefore plausible that the rheological properties of the particles formed after the gastric coagulation of milk can modulate appetite suppression, raising the potential for hot milk to promote satiety during the late stages of digestion when compared to cold milk.

When drawing physiological implications from *in vitro* data, the limitations in our semi-dynamic *in vitro* model system must be acknowledged. While efforts have been made to simulate many aspects of *in vivo* conditions, such as gastric mixing, emptying, and gastric juice production, this model does not entirely replicate the complexity of the *in vivo* situation. These differences may affect the translation of our results to the *in vivo* situation. Another difference that occurs *in vivo*, but not *in vitro* is the variation in the rate of gastric emptying due to consumption temperature. Several studies have examined the effect of consumption temperature on the rate of gastric emptying. With a carbohydrate beverage containing a known quantity of ^{13}C -sodium acetate, Fujihira et al. (2022) showed that a higher $^{13}\text{CO}_2$ percentage tended to be excreted in the breath when a hot beverage (60 °C) was consumed compared to a cold beverage (4 °C), suggesting a more rapid rate of gastric emptying at 60 °C. Using a comparable experimental design, Mishima et al. (2009) found that a liquid meal consumed at 60 °C was emptied significantly more rapidly in comparison to the same meal consumed at 4 °C. Sun et al. (1988) also reported that orange juice was emptied significantly faster when drunk hot vs cold. All these studies therefore suggest that a hot meal can be emptied more quickly from the stomach than the equivalent cold meal. Because the magnitude of this effect remains limited, and because we decided to adhere to the standardised international consensus for *in vitro* semi-dynamic digestion (Mulet-Cabero, Egger, et al., 2020), differences in gastric emptying rates were not considered in the present study. Consequently, caution must be taken when extrapolating the observed effects from our semi-dynamic model to *in vivo* settings, and future *in vitro* experimentation should consider accounting for this observed difference. Since MRI is suitable for studying digestion in human studies, another major perspective of this work would be to carry out *in vivo* MRI experiments and to test in this way the physiological relevance of these results obtained *in vitro*. Our results, with the compromises that must be made, pave the way for MRI monitoring of the *in vivo* kinetics of protein coagulation and particle creaming under different conditions.

5. Conclusion

In this study, we used MRI for *in vitro* investigation of the effects of consumption temperature on the gastric behaviour and digestion kinetics of whole milk. The results obtained have highlighted two key differences when milk is consumed cold *versus* when milk is consumed warm and hot. Firstly, we have confirmed that the coagulation of milk in the gastric phase is delayed when milk is consumed cold compared to when milk is consumed warm or hot. Secondly, we have shown in an original way that, although gastric temperature conditions only vary for the first half an hour of digestion, the creaming kinetics of particles appeared to be affected up to the end of digestion. When hot milk is consumed, dairy particles remain sediment at the bottom of the digestion vessel in contrast to when milk is consumed cold, where particles float to the top in the middle of digestion. These differences indicate that

variations in terms of gastric emptying rates of lipids, and possibly satiety, may be affected by the temperature at which milk is consumed. As a result, there is a need for additional *in vivo* MRI investigations to study the impact of milk consumption temperature on gastric digestion. This also raises further questions regarding the effect of consumption temperature on other foodstuff that behaves differently to milk during digestion, such as carbohydrate rich foods.

CRediT authorship contribution statement

Conor J. Fitzpatrick: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Maja Musse:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Jiajun Feng:** Methodology, Investigation. **Guylaine Collewet:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Data curation. **Tiphaine Lucas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Mark Timlin:** Resources. **Sylvain Challos:** Validation, Software, Data curation. **Stephane Quellec:** Validation, Supervision, Methodology, Investigation. **Didier Dupont:** Supervision, Conceptualization. **André Brodkorb:** Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Daniela Freitas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Steven Le Feunteun:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Acknowledgements

Conor Fitzpatrick and Andre Brodkorb are funded by Science Foundation Ireland (SFI) and the Department of Agriculture, Food and Marine on behalf of the Government of Ireland under Grant Number [16/RC/3835] - Vista Milk. Jiajun Feng is funded by a Chinese government scholarship (CGS). Daniela Freitas is funded by Science Foundation Ireland and the Irish Research Council (grant number 21/PATH-S/9418). This study was also financially supported by the Irish Research Council and the French ministries MEAE and MESRI through a PHC ULYSSES 2021 grant. The authors acknowledge Food for Health Ireland (FHI) for supplying the milk powders used in this trial. Part of this work has been performed using the PRISM core facility (Biogenouest, Univ Rennes, Univ Angers, INRAE, CNRS, France). Graphical Abstract was created with [Biorender.com](https://biorender.com).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2024.109864>.

References

- Ahlborn, N. G., Montoya, C. A., Hodgkinson, S. M., Dave, A., Ye, A., Samuelsson, L. M., et al. (2023). Heat treatment and homogenization of bovine milk loosened gastric curd structure and increased gastric emptying in growing pigs. *Food Hydrocolloids*, 137, Article 108380.
- Berglund, J., Ahlström, H., & Kullberg, J. (2012). Model-based mapping of fat unsaturation and chain length by chemical shift imaging—phantom validation and in vivo feasibility. *Magnetic Resonance in Medicine*, 68(6), 1815–1827.
- Brier, S., Maria, G., Carginale, V., Capasso, A., Wu, Y., Taylor, R. M., et al. (2007). Purification and characterization of pepsins A1 and A2 from the Antarctic rock cod *Trematomus bernacchii*. *FEBS Journal*, 274(23), 6152–6166.
- Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., et al. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, 14(4), 991–1014.
- Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using o-phthalaldehyde for determination of proteolysis in milk and isolated milk Proteins1. *Journal of Dairy Science*, 66(6), 1219–1227.
- Clark, G., Jamieson, I., Hinder, R., Polishuk, P., DeMeester, T., Gupta, N., et al. (1993). The relationship between gastric pH and the emptying of solid, semisolid and liquid meals. *Neuro-Gastroenterology and Motility*, 5(4), 273–279.
- Deng, R., Seimys, A., Mars, M., Janssen, A. E. M., & Smeets, P. A. M. (2022). Monitoring pH and whey protein digestion by TD-NMR and MRI in a novel semi-dynamic in vitro gastric simulator (MR-GAS). *Food Hydrocolloids*, 125, Article 107393.
- Fujihiro, K., Takahashi, M., Shimamura, K., & Hayashi, N. (2022). Effects of different temperatures of carbohydrate-protein-containing drinks on gastric emptying rate after exercise in healthy young men: Randomized crossover trial. *Journal of Physiological Anthropology*, 41, 37.
- Hamosh, M., Bitman, J., Wood, D. L., Hamosh, P., & Mehta, N. (1985). Lipids in milk and the first steps in their digestion. *Pediatrics*, 75(1), 146–150.
- Hernando, D., Kellman, P., Haldar, J. P., & Liang, Z. P. (2010). Robust water/fat separation in the presence of large field inhomogeneities using a graph cut algorithm. *Magnetic Resonance in Medicine*, 63(1), 79–90.
- Jacobs, W., Johns, P., Haselberger, P., Thompson, J. J., Sullivan, D., & Baugh, S. (2019). Calculation of whey protein fraction in milk-based infant formula: First action 2012.07. *Journal of AOAC International*, 96(3), 502–507.
- Lorieau, L., Halabi, A., Ligneul, A., Hazart, E., Dupont, D., & Floury, J. (2018). Impact of the dairy product structure and protein nature on the proteolysis and amino acid bioaccessibility during in vitro digestion. *Food Hydrocolloids*, 82, 399–411.
- Lucey, J. A., & Singh, H. (1997). Formation and physical properties of acid milk gels: A review. *Food Research International*, 30(7), 529–542.
- Luo, N., Ye, A., Wolber, F. M., & Singh, H. (2021). Effect of gel structure on the in vitro gastrointestinal digestion behaviour of whey protein emulsion gels and the bioaccessibility of capsaicinoids. *Molecules*, 26(5), 1379.
- Lüthi-Peng, Q., & Puhan, Z. (1999). Determination of protein and casein in milk by fourth derivative UV spectrophotometry. *Analytica Chimica Acta*, 393(1), 227–234.
- Mackie, A. R., Rafiee, H., Malcolm, P., Salt, L., & van Aken, G. (2013). Specific food structures suppress appetite through reduced gastric emptying rate. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 304(11), G1038–G1043.
- Mariette, F. (2009). Investigations of food colloids by NMR and MRI. *Current Opinion in Colloid & Interface Science*, 14(3), 203–211.
- Mat, D. J. L., Cattenoz, T., Souchon, I., Michon, C., & Le Feunteun, S. (2018). Monitoring protein hydrolysis by pepsin using pH-stat: In vitro gastric digestions in static and dynamic pH conditions. *Food Chemistry*, 239, 268–275.
- Mayar, M., Smeets, P., van Duynhoven, J., & Terenzi, C. (2023). In vitro 1H MT and CEST MRI mapping of gastro-intestinal milk protein breakdown. *Food Structure*, 36, Article 100314.
- McArthur, K. E., & Feldman, M. (1989). Gastric acid secretion, gastrin release, and gastric emptying in humans as affected by liquid meal temperature. *American Journal of Clinical Nutrition*, 49(1), 51–54.
- McMahon, D. J., & Oommen, B. (2008). Supramolecular structure of the casein micelle. *Journal of Dairy Science*, 91(5), 1709–1721.
- Mishima, Y., Amano, Y., Takahashi, Y., Mishima, Y., Moriyama, N., Miyake, T., et al. (2009). Gastric emptying of liquid and solid meals at various temperatures. *Journal of Gastroenterology*, 44(5), 412–418.
- Mulet-Cabero, A.-I., Egger, L., Portmann, R., Ménard, O., Marze, S., Minekus, M., et al. (2020). A standardised semi-dynamic in vitro digestion method suitable for food – an international consensus. *Food & Function*, 11(2), 1702–1720.
- Mulet-Cabero, A.-I., Mackie, A. R., Wilde, P. J., Fenelon, M. A., & Brodkorb, A. (2019). Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk. *Food Hydrocolloids*, 86, 172–183.
- Mulet-Cabero, A.-I., Rigby, N. M., Brodkorb, A., & Mackie, A. R. (2017). Dairy food structures influence the rates of nutrient digestion through different in vitro gastric behaviour. *Food Hydrocolloids*, 67, 63–73.
- Musse, M., Le Feunteun, S., Collewet, G., Ravilly, M., Quellec, S., Ossemond, J., et al. (2023). Quantitative magnetic resonance imaging of in vitro gastrointestinal digestion of a bread and cheese meal. *Food Research International*, 169, Article 112821.
- Nájera, A. De Renobales, M., & Barron, L. (2003). Effects of pH, temperature, CaCl₂ and enzyme concentrations on the rennet-clotting properties of milk: A multifactorial study. *Food Chemistry*, 80(3), 345–352.
- Picaud, J., Collewet, G., & Idier, J. (2016). Quantification of mass fat fraction in fish using water-fat separation MRI. *Magnetic Resonance in Imaging*, 34(1), 44–50.
- Piper, D. W., & Fenton, B. H. (1965). pH stability and activity curves of pepsin with special reference to their clinical importance. *Gut*, 6(5), 506–508.

- Salelles, L., Flourey, J., & Le Feunteun, S. (2021). Pepsin activity as a function of pH and digestion time on caseins and egg white proteins under static in vitro conditions. *Food & Function*, 12(24), 12468–12478.
- Sams, L., Paume, J., Giallo, J., & Carrière, F. (2016). Relevant pH and lipase for in vitro models of gastric digestion. *Food & Function*, 7(1), 30–45.
- Smoczyński, M. (2017). The effect of temperature on in vitro digestibility, fat globular size and free fatty acid bio-availability in milk fat. *Acta Sci Pol Technol Aliment*, 16(2), 127–134.
- Soop, M., Nehra, V., Henderson, G. C., Boirie, Y., Ford, G. C., & Nair, K. S. (2012). Coingestion of whey protein and casein in a mixed meal: Demonstration of a more sustained anabolic effect of casein. *American Journal of Physiology - Endocrinology And Metabolism*, 303(1), E152–E162.
- Sun, W. M., Houghton, L. A., Read, N. W., Grundy, D. G., & Johnson, A. G. (1988). Effect of meal temperature on gastric emptying of liquids in man. *Gut*, 29(3), 302–305.
- Synek, V. (2008). Evaluation of the standard deviation from duplicate results. *Accreditation and Quality Assurance*, 13(6), 335–337.
- Timlin, M., Brodkorb, A., Harbourn, N., O'Callaghan, T.F., Drouin, G., & Pacheco-Pappenheim, S., et al. The impact of seasonal calving systems with varying pasture allowance on Cheddar cheese composition, nutritional quality and ripening characteristics. Unpublished manuscript.
- Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2022). Kinetics of pepsin-induced hydrolysis and the coagulation of milk proteins. *Journal of Dairy Science*, 105(2), 990–1003.
- Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Effect of ingestion temperature on the pepsin-induced coagulation and the in vitro gastric digestion behavior of milk. *Food Hydrocolloids*, 139, Article 108550.
- Ye, A., Cui, J., & Singh, H. (2011). Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk. *Journal of Dairy Science*, 94(6), 2762–2770.