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Interfacial composition of coenzyme Q10 emulsions impacts coagulation of fortified milk during gastric digestion☆

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ABSTRACT

This study aimed to investigate the gastric digestion behaviour of heat-treated enriched milk containing Coenzyme Q10-loaded emulsions with different interfacial compositions. Four enriched milk types were compared: pasteurized with a Tween 80 stabilised emulsion (PAST-TW80), or with a sodium caseinate-stabilised emulsion (PAST-NaCN), and UHT with a TW80-stabilised emulsion (UHT-TW80), or PAST with a NaCN-stabilised emulsion (UHT-NaCN); all loaded with Coenzyme Q10. An *in vitro* dynamic gastric digestion model (Human Gastric Simulator) was utilized and the kinetics of milk coagulation and emptying of protein, lipid and Coenzyme Q10 were monitored. Adding NaCN-stabilised emulsion to heated milk led to a largely fragmented curd with signs of extensive droplets coalescence, disintegrating rapidly and accelerating protein and lipid release. Heated milk with TW80-stabilised emulsion produced a compact and closely integrated curd with limited coalescence, slowing nutrient emptying. UHT milk showed more curd fragmentation than PAST milk, regardless of emulsion type. The release profiles of Coenzyme Q10 were similar between UHT-TW80 and PAST-TW80 or between PAST-NaCN and UHT-NaCN, indicating the emulsion's interfacial composition as a key factor in controlling lipophilic bioactive release from the food matrix, regardless of heat treatment. These findings demonstrate that the emulsion's interfacial composition (NaCN vs TW80) and the heat treatment (PAST vs UHT) can be combined as a strategy to modulate milk coagulation kinetics and the rate of nutrient delivery to the small intestinal stage. This study provides insights into the development of functional milk products fortified with lipophilic bioactive compounds, as well as strategies for optimizing the controlled release of these compounds upon consumption.

1. Introduction

An oil-in-water (O/W) emulsion is an effective tool for creating lipid-based structured delivery systems that carry lipophilic bioactive compounds (McClements et al., 2007). The emulsion contains an oil phase, an aqueous phase, and an interfacial layer, which is formed by adding emulsifiers. These emulsifiers range from small molecule surfactants (such as the Tween and Span series) to surface-active biopolymers (such as proteins and polysaccharides). They provide various interfacial properties (thickness, charge, rheology, polarity, and interactions) that stabilize the emulsion, thereby protecting the bioactive compounds (McClements et al., 2009). In food industry, these bioactive compounds encapsulated in O/W emulsion can either be directly used as nutraceuticals through oral delivery or incorporated into food products to develop value-added fortified foods. Incorporating the emulsions into food products enables the homogeneous distribution of bioactive compounds throughout the food matrix and improve their stability and bioavailability (Lavelli et al., 2021).

Within the gastrointestinal tract, the physical stability of the emulsions highly depends on the type of emulsifiers applied. For example, protein-based emulsifiers are susceptible to pH changes and the presence of protease in the gastrointestinal (GIT) environment, while small molecule surfactant remain inert (Tan & McClements, 2021). These interfacial properties of emulsions during GIT digestion can directly affect the rate of release, bioaccessibility, and bioavailability of the encapsulated bioactive compounds. Another key factor influencing nutrient release and absorption is the food matrix in which emulsions are dispersed (Guo et al., 2020). Studies have shown that interfacial composition, oil properties, and the viscoelastic properties of the surrounding matrix each play a role in modulating gastric digestion, whether *in vitro* or *in vivo* (Liu et al., 2016; Soukoulis, Fisk, Gan, & Hoffmann, 2016; Steingoetter et al., 2015).

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Gastric digestion is a critical step in the uptake of nutrients from foods because it is where most of the food restructuring and breakdown occurs; this is induced by shear, low pH, ionic strength, and enzymatic hydrolysis (Guo et al., 2020). The rate of food disintegration in the stomach plays a crucial role in defining other processes such as gastric emptying and nutrient absorption (Bornhorst & Paul Singh, 2014). For instance, in the gastric environment, milk undergoes a drastic physical change from liquid to semi-solid, forming a so-called 'curd' (Ye et al., 2016). These curds can entrap fat globules and then gradually break down due to protein hydrolysis and mechanical shearing, leading to the release of fat globules and protein into the liquid fraction of the digestive contents (Ye et al., 2016). The formation of curds with different structures (dense vs. loose; cohesive vs. fragmented) affects the rate of proteolysis and consequently results in different rates of fat globule release from the curds into the digesta (Ye et al., 2016). Therefore, curd formation and disintegration in the stomach acts as a controlled delivery mechanism of proteins and lipids to the small intestine. It has been described that processing steps such as heat treatment and homogenisation of milk lead to the formation of a soft curd with loose structure that is more rapidly hydrolysed by pepsin compared with unheated milk (Ye, Cui, Dalgleish, & Singh, 2017; Ye et al., 2019).

The gastric colloidal behaviour of the components in milk, especially milk proteins, can be utilized as tool to manipulate nutrient digestion in various dairy products and protein emulsions, including, but not limited to, infant formulae, solid cheeses and emulsion-type beverages (Ye, 2021). Therefore, milk can serve as an excellent medium for delivering bioactive substances to the GIT. Liu, Lei, Yuan, and Gao (2014) compared β-carotene release from four types of emulsions stabilized by different milk proteins (β-lactoglobulin, sodium caseinate, lactalbumin, and lactoferrin) using a static *in vitro* digestion model. They concluded that the emulsion interfacial composition determines the release at different digestive stages. Our group previously described the *in vitro* digestion behaviour of recombined milk systems fortified with a curcumin-loaded caseinate-stabilised nanoemulsion, showing that the physical properties of the food are a key factor in controlling the release of curcumin into the GIT fluids (Qazi et al., 2022). In another study, we found the bioavailability of coenzyme Q10 (CoQ10) *in vivo* was significantly higher when encapsulated into a modified starch-stabilised nanoemulsion and added to a milk protein beverage (Niu et al., 2020). However, studies on how food structure affects the release of bioactive compounds during the GI tract are very limited. Furthermore, how various emulsion interfacial compositions interact with milk and the influence the kinetics of food disintegration and the release of lipophilic bioactive compounds during digestion remains unknown.

This study aimed to systematically explain the influence of varying: (1) the interfacial composition of CoQ10-loaded emulsions (non-interacting or interacting emulsifiers) and, (2) milk heat treatment (pasteurisation vs UHT) on the rate of gastric milk coagulation and release of nutrients and CoQ10. Polysorbate 80 (TWEEN 80), a non-ionic surfactant, was selected as a non-interacting emulsifier, while sodium caseinate, a protein-based emulsifier, was chosen as an interacting emulsifier due to their distinct molecular structures and emulsification mechanisms. This investigation provides valuable insights into how interactions between emulsifier composition and heat treatments affect the disintegration of fortified milk in the stomach and the release of lipophilic bioactive compounds.

2. Materials and methods

2.1. Materials

Coenzyme Q10 (CoQ10) was purchased from Pure Nature (Auckland, New Zealand). Sodium caseinate (sodium caseinate 180) was purchased from Fonterra Co-operative Group Limited (Auckland, New Zealand). According to the manufacturer, this ingredient had a protein content of 92.3 %, fat content of 0.6 %, ash content of 4 % and moisture content of 4.83. Digestive enzymes such as pepsin from porcine gastric mucosa lyophilized powder (EC 3.4.23.1, P7012; ≥2500 units/mg protein) and lipase from *Candida rugosa* (EC 3.1.1.3, L1754; type VII, ≥700 unit/mg solid), and polysorbate 80 (TWEEN 80, W291706) were purchased from Sigma-Aldrich New Zealand Co. (Auckland, New Zealand). The raw milk was purchased from a local farm (Palmerston North, New Zealand). Corn oil was purchased from a local supermarket (Davis Trading Company, Palmerston North, New Zealand). Hydrochloric acid, (HCl, 36.5–38 %) was purchased from Thermo Fisher Scientific New Zealand (Auckland, New Zealand). All other chemicals were analytical grade from Sigma-Aldrich. Milli-Q water (Millipore Corporation, Bedford, MA, USA) was used for all experiments.

2.2. Preparation of CoQ10-loaded emulsions

The CoQ10-loaded emulsions were composed by 20 wt% of the oil phase containing CoQ10 and 80 wt% of the aqueous phase containing sodium caseinate (NaCN) or Tween 80 (TW80). The oil concentration (20 wt%) was rationally selected to encapsulate a high CoQ10 payload and ensure accurate quantification of the substance in digesta fractions. To prepare the oil phase, CoQ10 were dissolved in corn oil at 5 wt%, 40 °C. The aqueous phases were prepared by dissolving NaCN or TW80 at a concentration of 1 wt% in Milli-Q water. The emulsifier concentration was selected based on preliminary trials testing concentrations from 0.5 to 2 wt%. A 1 wt% emulsifier produced CoQ10 emulsions with similar oil droplet sizes for both types of emulsifiers. The combined oil and water solution were mixed using a high shear mixer (L5M, Silverson, Massachusetts, USA) at 9000 rpm for 3 min to form a coarse emulsion. The coarse emulsion was then passed through a microfluidizer M−110P (Microfluidics Corporate, MA, USA) at 50 MPa, two passes. The stock emulsions containing 20 wt% oil, 1 wt% CoQ10 and 1 wt% NaCN or TW80 were stored at 4 °C for 1 day before further use.

2.3. Particle size analysis of CoQ10 loaded emulsion

The particle size and size distributions of CoQ10-loaded emulsions were analysed using a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) with an automated small volume sample dispersion unit (Hydro 2000S). The refractive index of the emulsion was set to 1.47, while that of water is 1.33. The droplet size was reported as the volume-weighted mean diameter D [4,3] (μm). Particle size distributions were obtained by measuring oil droplet size as a function of volume frequency. Both TW80- and NaCN-stabilized emulsions exhibited a monomodal particle size distribution (Fig. S1). The D [4,3] value of the TW80-stabilized emulsion and the NaCN-stabilized emulsion was 0.536 and 0.533, respectively. Despite the use of different emulsifiers, the volume-weighted particle size D [4,3] was not significantly different between the two CoQ10-loaded emulsions ($p = 0.46$). This ensured a similar oil droplet size in the CoQ10-loaded emulsions with different interfacial compositions, facilitating their subsequent incorporation into various heat-treated milks to form CoQ10-enriched milk.

2.4. Heat treatment of milk and preparation of CoQ10-enriched milk

The milk treatment was carried out in the pilot plant facilities of Massey University (FoodPilot, Massey University, Palmerston North, New Zealand). Twenty litres of raw milk were processed using a milk skimmer (Type 106AE, Alpha Laval, New Zealand). Subsequently, the skimmed milk underwent either pasteurization (at 72 °C for 15 s) or Ultra High Temperature (UHT) treatment (at 140 °C for 4 s) using a UHT unit, working at 100 L/h. Milk was aseptically filled into 250 mL white plastic bottles. The proximate composition of the UHT and pasteurized skimmed milk was determined using a milk analyser (MilkoScan FT1, FOSS, Denmark) (Table S1). The fat contents of both types of milk were 0.1 wt% with no significant difference between the heat treatments (Table S1). The pasteurized milk was stored at 4 °C, while the UHT-

treated sample was stored at room temperature (22 °C) for future use.

CoQ10-enriched milk was prepared by blending the CoQ10 stock emulsion (NaCN/TW80) with the pasteurized/UHT milk at a ratio of 1:3 (w/w) and mechanically stirring for 20 min. The CoQ10-enriched milk achieved a final oil concentration of 5 wt% and a CoQ10 concentration of 0.25 wt%. Freshly prepared fortified milk samples were used for digestion experiments. The samples were labelled as PAST-TW80, PAST-NaCN, UHT-TW80 and UHT-NaCN to represent pasteurized milk incorporated with Tween 80 stabilized CoQ10 emulsion, pasteurized milk incorporated with sodium caseinate stabilized CoQ10 emulsion, UHT milk incorporated with Tween 80 stabilized CoQ10 emulsion and, UHT milk incorporated with sodium caseinate stabilized CoQ10 emulsion, respectively.

2.5. In vitro dynamic gastric digestion

A Human Gastric Simulator (HGS) was used to perform *in vitro* dynamic gastric digestion of CoQ10-enriched milk samples. The HGS is composed of a latex chamber, a driving system with rollers to simulate peristaltic contractions, a peristaltic pump to inject simulated gastric fluid (SGF) with pepsin, and a simulated pyloric valve to empty digesta Kong and Singh (2010).

A 200 g of CoQ10-enriched milk sample containing 500 mg CoQ10 (suggested standard dosages for CoQ10 supplements ranging between 60 and 500 mg daily, WebMD) was used as food for *in-vitro* gastric digestion experiment. The simulated gastric fluid (SGF) (pH 1.3) and the pepsin solution (4000 unit/ml) was prepared according to the INFOGEST standardized protocol (Brodkorb et al., 2019). CoQ10-fortified milk was mixed with SGF to a 1:1 ratio. Firstly, the "food (200 g)" was mixed with 20 g of SGF, pH 1.3 (10 %) to mimic the fasted state of the stomach, and remained at 37 °C in a water bath for 1 min. This mixture was then poured into the latex artificial stomach with simulated peristaltic contractions occurring 3 times per min. The remaining SGF and pepsin (180 g, 90 %) were added at a combined rate of 0.75 mL/min. The digesta was emptied every 30 min with 50 g collected. At different digestion intervals (30, 60, 90, 120, 150, 180, 210 and 240 min), samples were collected and adjusted to pH 7 with 1 M NaOH and/or 1 M HCl to stop the activity of pepsin. Samples for microscopy analyses were not pH adjusted and analysed immediately after sample collection.

2.6. pH, total solids, protein and lipid determination

The pHs of CoQ10-fortified milk samples were recorded before and after mixing with SGF. The initial pH in HGS was defined as the pH of the fresh samples before digestion, and the pH of the emptied digesta was recorded every 30 min, with the assumption that this corresponded to the pH within the HGS.

To determine the total solids content of the digesta collected at different digestion time intervals (30, 60, 90, 120, 150, 180, 210 and 240 min) and the curd obtained at the end of the digestion, samples were collected and dried at 105 °C overnight in a hot air oven. The dried mass of each sample was weighed, and the total solids retention in HGS was calculated as described in Eq. (1):

$$Total\ solids\ retention\ (\%) = \frac{W_0 - W_t}{W_0} \times 100 \quad (1)$$

where W_0 is the sum of dry weight of initial enriched milk, salts, and pepsin (g), and W_t is the cumulative dry weight of emptied digesta at time t (g).

Protein and fat content were conducted by Nutrition Lab at Massey University (Palmerston North, New Zealand). Crude protein was analyzed by Dumas method according to AOAC 968.06 with a N-P conversion index of 6.25. Fat content in the emptied digesta at different digestion periods was determined using the Mojonnier method as outlined in AOAC 989.04, which is typically employed for dairy products.

2.7. Characterization of curd disintegration

Curd formation/disintegration during gastric digestion was characterized through particle size distribution using the wet sieving method (Guo et al., 2015). For this purpose, the *in vitro* digestion process was stopped at 30, 45, 60, 120, 180, and 240 min. Then, the gastric contents (gastric chyme) were passed through a pre-weighted and oven-dried stack of sieves (0.038, 0.50, 0.85, 1.40, 2.00 and 3.15 mm). Each sieve was washed carefully using a mild spray bottle. Carefully wiping out the free water remained in each sieve, the wet weight of each sieve was measured to get the wet weight of curds in each sieve. The particle size distribution was plotted as a cumulative wet weight percentage across all sieve sizes at each digestion time. The wet weight percentage for each sieve size and digestion time was calculated as described in Eq. (2):

$$Wet\ weight(\%) = \frac{wet\ weight\ of\ curds\ in\ each\ sieve\ (g)}{Total\ weight\ of\ food(g)} \times 100 \quad (2)$$

2.8. Microstructure analysis by CLSM

The microstructure of the curd with digesta obtained from the digestion of CoQ10-enriched milk after 240 min was investigated using a confocal laser scanning microscope (CLSM) (Model Zeiss LSM900 with Airyscan 2, Carl Zeiss, Jena, Germany). Sample preparation used the double staining method described by Gallier, Gragson, Jimenez-Flores, and Everett (2010) to mark protein and lipid, respectively. Protein was stained with a fast green solution (1 mg/mL) illuminated by a He–Ne laser with an excitation line at 633 nm and collected between 638 and 750 nm, while lipid droplets were stained with a Nile red solution (1 mg/mL) illuminated by an argon laser with an excitation line at 488 nm and collected between 494 and 605 nm. Mixtures of curd and digesta were stained with both stain solutions for 15 min, respectively, and placed on a concave microscope slide covered with a coverslip. They were then observed under the microscope with the 63 × oil immersion objective lens (HCX PL APO Lambda Blue, 63.0 × 1.40 oil UV, Leica Microsystems, Wetzlar, Germany) at room temperature. Representative images were captured with Zeiss ZEN 3.1 (Blue Edition) imaging software (Carl Zeiss, Germany) and were analyzed using ImageJ (National Institute of Health, Bethesda, MD, USA).

2.9. Coenzyme Q10 quantification

Coenzyme Q10 (CoQ10) quantification was performed using). Samples fortified with CoQ10 emulsion before and after digestion were analysed. Firstly, CoQ10 was extracted from the milk matrix by the following steps. One gram of undigested or digested sample was added to 1.25 mL of phosphate buffer (0.8 M, pH 7.4) and vortexed. Then, 0.25 g of lipase powder was added and vortexed until well dispersed. Samples were incubated at 37 °C (±2 °C) for 1 h, under shaking at 100 rpm. Samples were removed after the incubation time and once cooled to room temperature, 2.5 mL of alcohol (EtOH/MeOH, 95:5) and 0.25 g of potassium carbonate was added to stop the reaction. CoQ10 was extracted adding 3 mL of hexane with thorough mixing, followed by centrifugation (3000g, 10 min). The hexane fraction was collected in a separate tube. The extraction procedure was repeated three times. Then, all three hexane fractions were combined and dried using a Nitrogen evaporator (Reacti-Therm I TS-18822, Thermal Fisher Scientific, Rockwood, TN, USA). The dried fraction was re-dissolved in 5 mL of ethanol. The sample was filtered through a 0.22 μm syringe membrane and further diluted ten-fold for analysis.

The samples were analysed using a High-Performance Liquid Chromatography (HPLC) (Agilent 1200 series, Santa Clara, CA), fitted with a

300A Jupiter® C18 column (150 mm × 4.6 mm, 5 μm, Phenomenex, Torrance, CA). The mobile phase consisted of MeOH: absolute EtOH (75:25 v/v), and was run at a flow rate of 1.0 mL/min under isocratic conditions at 25 °C. The UV detector was set at 275 nm. Five microliters (5 μL) of the sample was injected, and the CoQ10 concentration was quantified using a standard curve. The standard curve was established using a CoQ10 ethanol solution with concentrations of 25, 50, 100, 150, and 200 μg/mL.

2.10. Statistical analysis

Duplicate HGS digestions were performed independently, with overlapping results confirming the consistency of the findings. Triplicate analyses were conducted, determining the average and standard deviation of total solids, protein content, lipid content, and CoQ10 concentration. The statistical analysis was conducted using SPSS software (IBM, USA). This included employing the Student's *t*-test to compare differences between two groups: UHT versus Pasteurized milk and CoQ10-loaded emulsions stabilized by TW80 versus NaCN. Additionally, one-way ANOVA was utilized for a comparative analysis of four CoQ10 enriched samples. Pearson correlation analysis was performed to explore the relationships between lipid content and CoQ10 concentration, as well as between protein retention and lipid retention in the digesta.

3. Results and discussion

3.1. pH profiles of CoQ10-enriched milk during gastric digestion

All the CoQ10-enriched milk samples exhibited a similar pH of 6.8 before digestion (Fig. 1). During gastric digestion, the pH of the CoQ10-enriched milk samples gradually decreased to around 2.0 in the HGS environment over 240 min (Fig. 1), a result of the continuous addition of SGF.

Pasteurization and UHT treatments affected the rate of pH decrease in non-interacting TW80-stabilized samples, with UHT milk showing a slower decrease in pH with digestion time than the pasteurized milk (Fig. 1). This observation aligns with the findings of Ye et al. (2019), who compared pH changes among unheated, pasteurized, and UHT milk during gastric digestion. UHT milk had a slower decrease in pH with digestion time than the unheated and pasteurized milk. However, this difference was not observed between PAST-NaCN and UHT-NaCN (Fig. 1). In fact, UHT-NaCN exhibited a steep decline in pH, reaching the lowest value of 1.4 compared to the other samples (Fig. 1). This steep decrease in pH observed in UHT-NaCN could be attributed to the high degree of curd disintegration and protein hydrolysis, which lowers the buffering capacity of the chyme. This will be discussed further in the following section. These results suggest that NaCN could interact with the milk matrix in a way that leads to changes in pH profiles affected by heat treatments.

Regardless of the heat treatments, the milk samples enriched with the emulsion stabilized by NaCN showed a slower pH decrease within 180 min of digestion compared to those emulsified with TW80 (Fig. 1), indicating that the emulsions stabilized with different emulsifiers did impact the pH profiles of the CoQ10-enriched milk. Additional protein content used in the preparation of NaCN stabilised emulsions provided a higher buffering capacity causing a slower drop in pH. Similar results have been reported for a whey protein-stabilized emulsion, a whey protein-stabilized emulsion with additional sodium caseinate, a Tween 80-stabilized emulsion, and homogenized full-fat milk, where authors noted that the fastest pH drop during *in vitro* gastric digestion was found in Tween 80 systems (van Aken, Bomhof, Zoet, Verbeek, & Oosterveld,

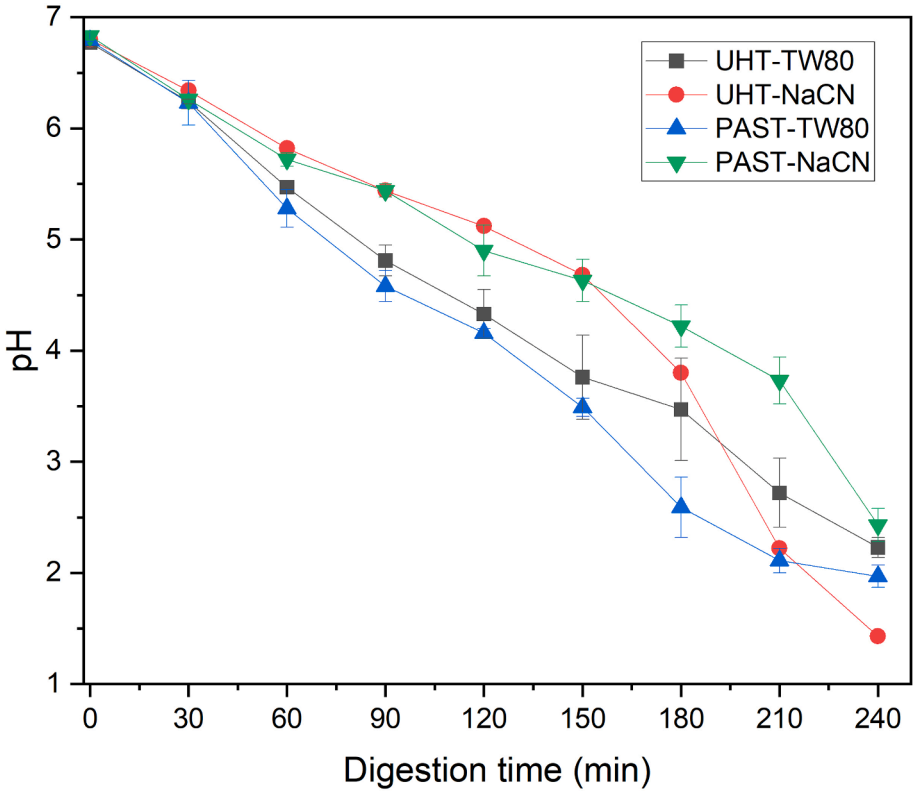

Fig. 1. Change in pH of Co-enzyme Q10 (CoQ10) enriched milk during gastric digestion in the human gastric simulator. PAST-TW80 (■): Pasteurized milk fortified with Co-Q10, emulsified using Tween 80. PAST-NaCN (●): Pasteurized milk fortified with Co-Q10, emulsified with sodium caseinate.UHT-TW80 (▼): UHT-TW80: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified with Tween 80. UHT-NaCN (▲): Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified with sodium caseinate.

2011).

3.2. Gastric behaviour of CoQ10-enriched milk

Protein coagulation occurs in milk within the initial 10 min when the pH drops to 6.2 under simulated dynamic gastric digestion conditions (Ye et al., 2019). This coagulation results in curd formation in milk, but the morphology of these curds varies depending on the pre-treatment of milk. The formation and disintegration of curds from four CoQ10-enriched milks were investigated by analysing the particle size distribution during gastric digestion, as well as their morphology and microstructure at the end of digestion.

3.2.1. Curd disintegration kinetics

The digestion behaviour curds were analysed by calculating the cumulative wet weight (%) of curds from all size sieves over various digestion times. As digestion time increased, the cumulative wet weight of curds from all samples consistently decreased (Fig. 2). Within the initial 30 min of digestion, the milk underwent both coagulation (within the first 10 min) and disintegration. However, the initial wet weight of the curd was not determined in this study. Therefore, the wet curd weight at 30 min was obtained after approximately 20 min of disintegration. At 30 min, the wet weights of NaCN curds (Fig. 2 B&D) were higher than those of TW80 curds (Fig. 2 A&C); additionally, UHT curds (Fig. 2 C&D) showed higher wet weights than PAST curds (Fig. 2 A&B). At the end of digestion, all the curds had a similar wet weight of around 7–9 % (w/w) (Fig. 2, 240 min). These results suggested that the curds from TW80 and NaCN, as well as those from PAST and UHT, had different structures under gastric conditions.

The disintegration behaviour was also characterized through particle size distribution of the curds. Curds formed through protein coagulation during gastric digestion were collected from the human gastric simulator (HGS) at various time intervals and passed through different sizes of sieves. All four samples exhibited unique particle size distribution. Samples with the same interfacial composition showed similarities in their particle size distributions. TW80 curds contained a major portion of large particles across all digestion times, with sizes over 3.15 mm (Fig. 2 A&C, yellow bar). In contrast, NaCN curds displayed a diversity of particle sizes, with smaller particles retained predominantly in sieves <1.4 mm (Fig. 2 B&D; grey, red, blue and green bar). The morphology of curds on the sieves showed that TW80 curds had a large portion of tight and close-knit networks, while NaCN curds contained more particulate and loose structures (Fig. S2). Heat treatment affected curd disintegration during the initial stage (first two hours), as evidenced by different curd morphologies on the sieves (Fig. S2) and varying size distribution profiles (Fig. 2) between the PAST and UHT samples. However, at the end of the digestion, these differences became less obvious.

The formation of structured curds is initiated by the action of the milk-clotting enzyme, pepsin, on κ-casein (Ye et al., 2016). The differences in the structure of the curds obtained between UHT and pasteurized milk have been observed by Ye et al. (2019). A similar, higher wet weight in UHT curds was due to a higher degree of hydration, with a

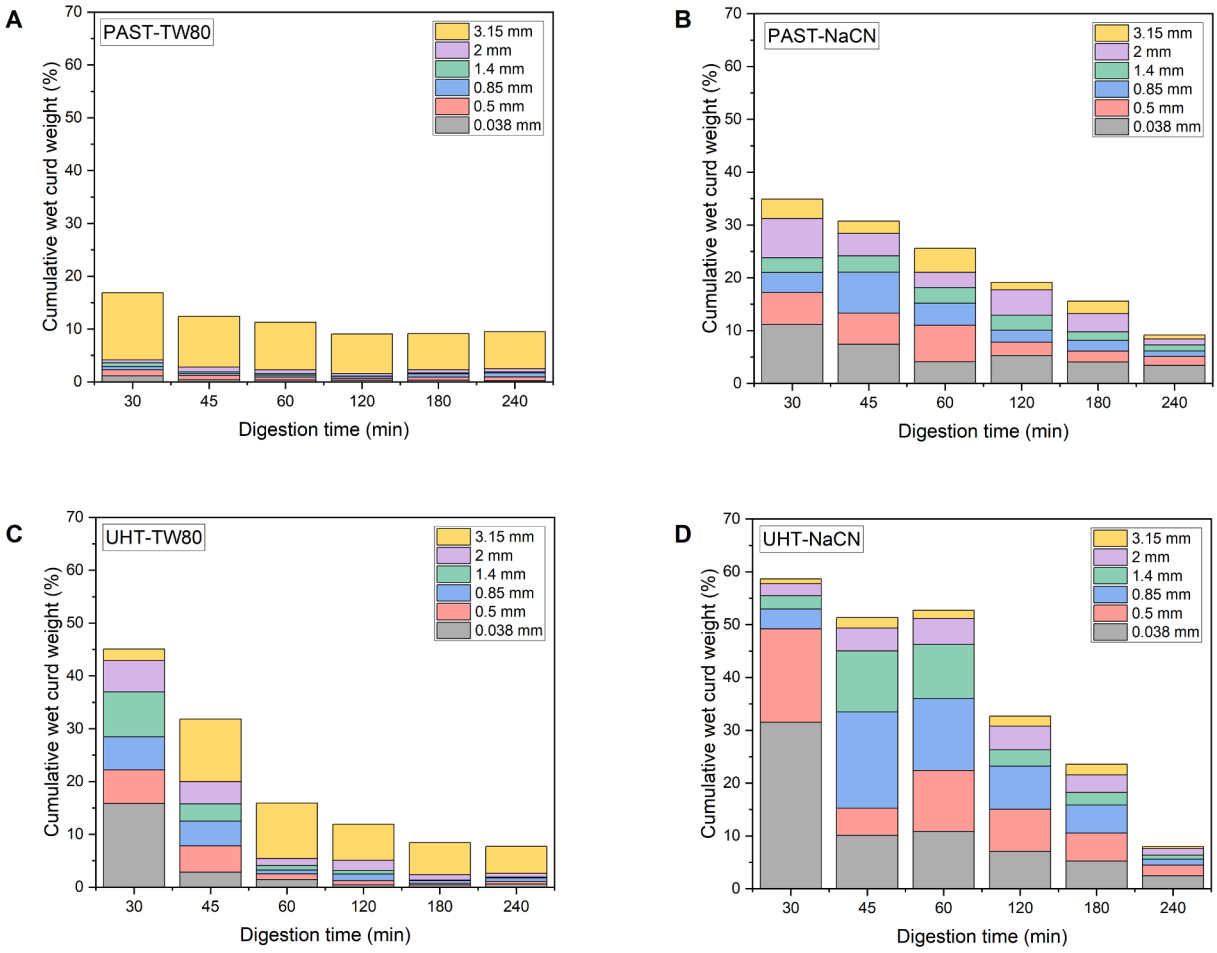

Fig. 2. Change in particle size distribution of curds from Co-enzyme Q10 (Co-Q10) enriched milk during gastric digestion. (A) PAST-TW80: Pasteurized milk fortified with Co-Q10, emulsified using Tween 80. (B) PAST-NaCN: Pasteurized milk fortified with Co-Q10, emulsified using sodium caseinate. (C) UHT-TW80: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using Tween 80. (D) UHT-NaCN: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using sodium caseinate.

structure featuring more and larger voids. This could be reflected in a larger portion of smaller-sized particles when passing through the sieves (Fig. 2). Between the TW80 and NaCN curds, the NaCN curds could also have a higher degree of hydration as well as a porous and looser structure. NaCN might be susceptible to the proteolytic activity of pepsin and interact with the milk matrix, while TW80 is inert under gastric conditions. The different structures of these curds subsequently influence the rates of protein hydrolysis by pepsin and the release of fat globules during curd disintegration (Ye et al., 2016).

3.2.2. Curd properties

Upon completion of the 240-minute digestion period, the remaining curds (gastric chyme) in the HGS were collected and compared based on their appearance and texture. All curds displayed a yellowish colour, indicating the presence of CoQ10 (Fig. 3). However, the curds formed from milk samples enriched with the emulsion stabilized by TW80 and NaCN are completely different. In general, TW80 curds are solid, while NaCN curds are soft and watery (Fig. 3). The PAST-TW80 curd exhibited a cohesive, dough-like texture with a relatively smooth surface. In contrast, the PAST-NaCN curd appeared as a semi-solid mass with a lumpy, irregular, and crumbly texture (Fig. 3). UHT-TW80 curds were chunky and fragmented, resembling curd, while UHT-NaCN curds displayed a lumpy and coarse texture with a soft and creamy consistency (Fig. 3).

These results suggest that the interfacial composition of emulsion droplets significantly influences curd formation and morphology, resulting in varied textures and structures. Cho, Lucey, and Singh (1999) compared rheological properties of acid milk gels made from heated recombined skim milk with fat globules stabilized by different emulsifiers. Their study demonstrated that non-interacting materials such as Tween act as a filler, while interacting materials such as NaCN and heated whey protein concentrate can reinforce the gel matrix, resulting in an increase in gel strength. In the gastric environment, the curd with CoQ10 emulsion stabilized by the interacting emulsifier NaCN is more susceptible to disruption through pepsin hydrolysis compared to those stabilized by TW80, therefore leading to a weak and soft structure. Therefore, we hypothesized that the different curd properties found in this study were mainly influenced by the type of emulsifier chosen in the CoQ10 emulsions. For instance, when gastric-driven interactions between an inactive filler (Tween-stabilised oil droplets) and milk proteins occur, lipid interfaces remain intact. As the pH of the gastric environment decreases and approaches caseins' isoelectric point, proteins aggregation occurs forming a uniform and closed-knitted clot. On the contrary, when an active filler (NaCN-stablised oil droplets) is added to milk, both the lipid droplets' interfaces and proteins in the matrix are susceptible to pepsin hydrolysis. Therefore, the clot structure changes because protein aggregation occurs in a rather random manner, leading to the formation of a highly fragmented clot.

3.2.3. Curd observed by CLSM

The curds formed by CoQ10 enriched milk at the end of gastric digestion were examined by confocal laser scanning microscope (CLSM) to visualize the distribution of proteins and lipids. Samples stabilized with TW80 showed a structure primarily composed of protein, with small, sparsely distributed oil droplets (Fig. 2). In contrast, the NaCN group displayed significant oil droplet coalescence, characterized by irregularities and larger pools of lipids within the protein network (Fig. 2). More oil droplets were observed in the curd matrix in NaCN samples, while fewer oil droplets were noted in Tween samples (Fig. 2), indicating that NaCN at the interface was more involved in clot formation due to the protein interface.

NaCN at the oil droplet interface is susceptible to being hydrolysed by pepsin, and then the interfaces become ruptured or damaged during digestion, inducing coalescence. Fat globule coalescence within the clot during gastric digestion has been previously observed *in vitro* in raw milk samples (Roy, Ye, Moughan, & Singh, 2021; Ye et al., 2019). The coalescence of fat globules is due to the rupture or removal of the proteins of the milk fat globule membrane (MFGM), which are caused by pepsin hydrolysis and simulated contraction movements (Le et al., 2012; Ye, Cui, & Singh, 2011). NaCN in this study acts in a similar function as the MFGM proteins. In addition, Liu et al. (2016) confirmed *in vivo* that acid-stable emulsions stabilized by polysorbate 80 largely maintained their stability throughout gastric digestion. In contrast, acid-unstable emulsions stabilized by NaCN underwent structural changes, leading to a less homogeneous intragastric fat distribution.

Regarding heat treatments, heat treatment impacted protein structure: PAST samples exhibited a denser and more cohesive protein network the fragmented and crumbly UHT samples (Fig. 2). Heat treatment causes whey proteins to denature and associate with casein micelles and fat globules, impairing the interactions between caseins and between casein and fat globules (Anema & Li, 2003; Ye et al., 2016). This alteration may modify the orientation of casein strands and lead to less complete fusion rearrangements, resulting in a more open and looser curd structure (Ye et al., 2019). As Ye et al. (2019) expected, this structural change was more pronounced in UHT milk than in PAST milk, aligning with the results observed in this study.

3.3. Gastric emptying behaviour of CoQ10-enriched milk

3.3.1. Total solids retention

The total solids retained in the HGS decreased over time, attributable to gastric emptying and protein hydrolysis. UHT-treated samples exhibited a faster rate of total solids emptying compared to PAST-treated samples (Fig. 4A), which may be attributed to the looser and more fragmented protein matrix in UHT-treated milk. This matrix is more susceptible to enzymatic hydrolysis, facilitating structure breakdown and subsequent release of more solids into the digesta upon emptying (Ye et al., 2016). At the end of digestion, PAST samples contained significantly higher total solids than the UHT samples in the HGS (Fig. 4A).

Regarding interfacial composition, TW80 samples demonstrated a quicker rate of total solid emptying compared to NaCN samples (Fig. 4A). This could be due to the additional protein at the interface and its interaction with the milk protein in NaCN samples, leading to slower digestion. However, this trend was not observed in UHT-NaCN. UHT-NaCN showed a slower emptying rate at the beginning of digestion but accelerated after 150 min, eventually resulting in much lower total solids content at the end of digestion compared to PAST-NaCN (Fig. 4A). The notable change in total solids in UHT-NaCN during digestion corresponded well with its pH change, with the lowest pH observed in UHT-NaCN at the end of digestion (Fig. 2). At lower pH, increased solubilization occurred, releasing more solids into the digesta. This effect could be ascribed to a combination of milk treatment and emulsifier.

3.3.2. Protein and lipid retention

Protein is one of the main factors influencing gastric emptying in milk system. Similar to the retention of total solids, UHT samples showed a faster decrease in protein content compared to PAST samples (Fig. 4B). NaCN samples also demonstrated a more rapid protein emptying rate than TW80 samples (Fig. 4B). Protein in UHT-NaCN even emptied to 0 % before the 240 min digestion (Fig. 4B). At the end of digestion, PAST-TW80 retained a significantly higher protein content in the HGS, followed by UHT-TW80 (Fig. 4B). These results were expected due to the different curd structures formed from the four milk samples. The looser, weaker, more fragmented curd structure results in faster hydrolysis and more rapid emptying of protein.

Lipids in CoQ10-enriched milk originated solely from the CoQ10-loaded oil-in-water emulsion. The retention of lipids followed the same pattern as total solids retention (Fig. 4 A&C). Lipid release occurred as a result of curd disintegration. This release was primarily influenced by the interfacial composition. TW80 samples exhibited highly overlapped lipid emptying patterns, regardless of milk treatments

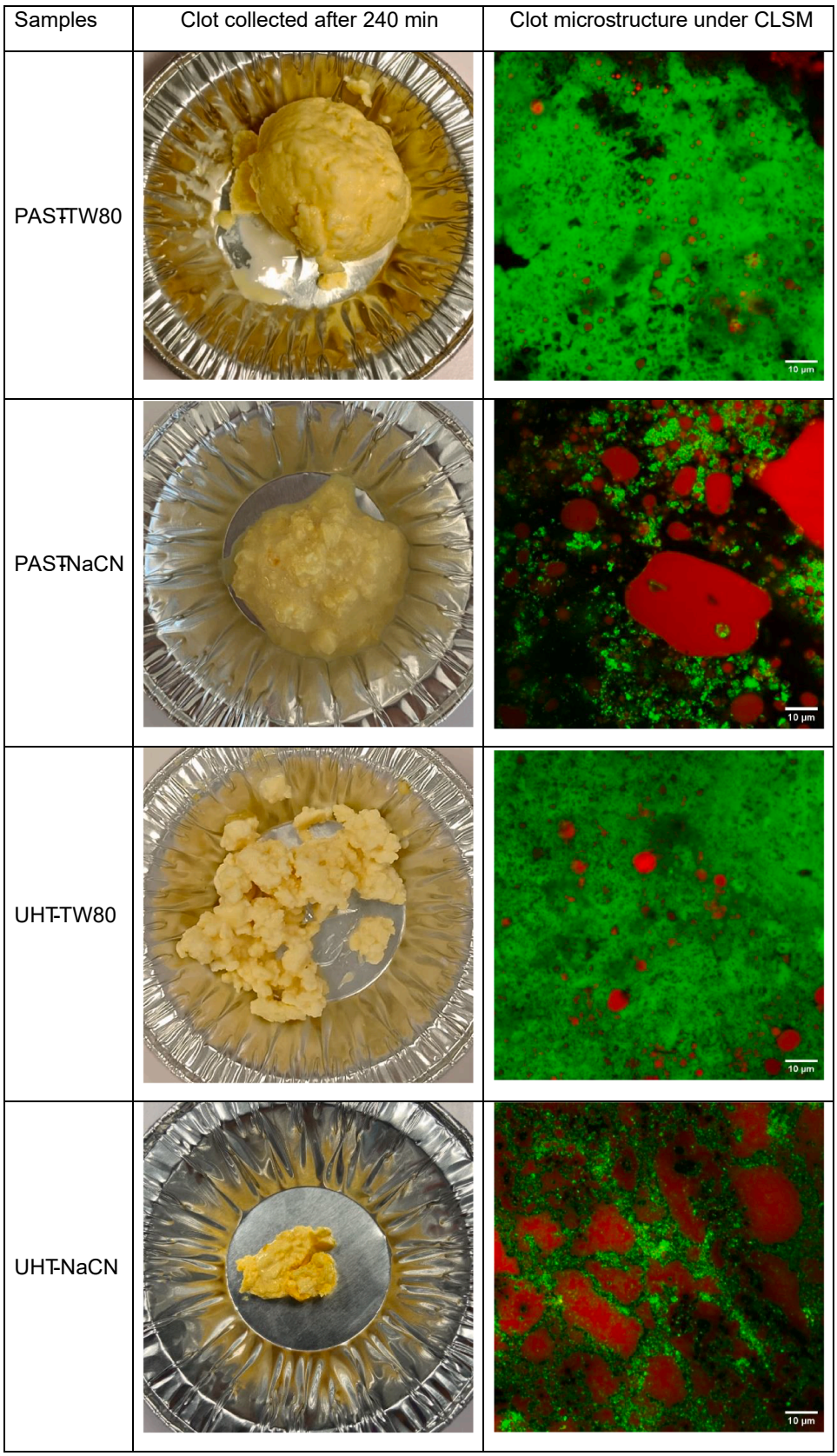

Fig. 3. Macrostructure and microstructure of curds from Co-enzyme Q10 (Co-Q10) enriched milk samples after 240 min of gastric digestion. PAST-TW80: Pasteurized milk fortified with Co-Q10, emulsified using Tween 80. Microstructure was detected by confocal laser scanning microscope. Green colour indicates protein while red colour indicates lipid present in the matrices. PAST-NaCN: Pasteurized milk fortified with Co-Q10, emulsified using sodium caseinate. UHT-TW80: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using Tween 80. UHT-NaCN: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using sodium caseinate.

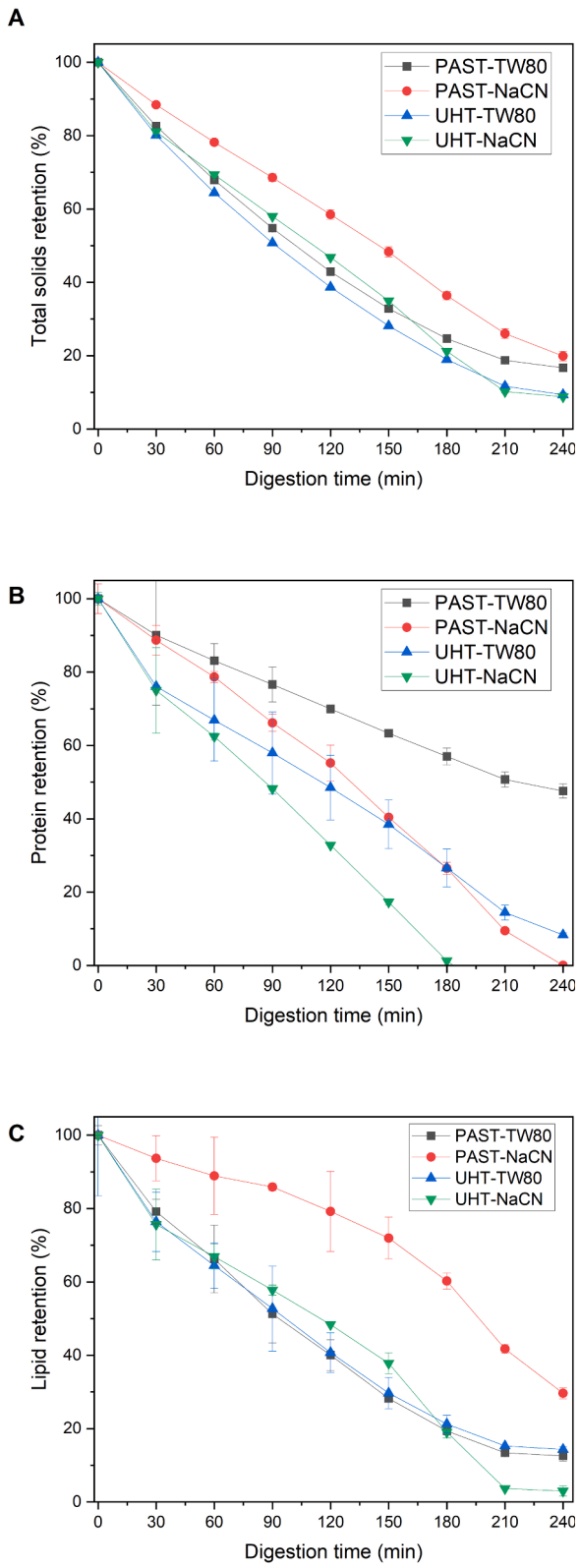

Fig. 4. Change in total solids (A), protein (B) and lipid (C) of gastric digesta from Co-enzyme Q10 (Co-Q10) enriched milk during gastric digestion. PAST-TW80: Pasteurized milk fortified with Co-Q10, emulsified using Tween 80. PAST-NaCN: Pasteurized milk fortified with Co-Q10, emulsified using sodium caseinate. UHT-TW80: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using Tween 80. UHT-NaCN: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using sodium caseinate.

(Fig. 4C), reinforcing the evidence that TW80 does not interact with the milk matrix. The release pattern was dependent on the breakdown of the protein structure, which was not significantly different as observed under CLSM. NaCN samples showed a completely different lipid release pattern from the TW80 samples. PAST-NaCN exhibited a significantly slower lipid release compared to the rest of the samples (Fig. 4C). However, UHT-NaCN displayed a slightly faster lipid release than the TW80 samples (Fig. 4C). These differences can be explained by the results of digestion behaviour of the curd structure (Section 3.2.3). NaCN was proposed as an interacting emulsifier and acts similarly to MFGM proteins at the interface of oil droplets. The oil droplets stabilized by NaCN underwent coalescence during digestion, as observed with large oil droplets trapped in the protein matrix under CLSM (Fig. 3), which results in more lipid retention in the stomach and slower lipid release in the PAST-NaCN sample (Fig. 4C).

3.4. Microstructure of emptied digesta by CLSM

To understand the distribution and release profile of protein and lipids in the HGS, the digesta of CoQ10-enriched milk was sampled at various digestion time periods and observed under CLSM. Before digestion, all four samples exhibited an even protein matrix with small oil droplets uniformly distributed (Fig. 5, time 0 min). During digestion, a significant difference in protein structure emerged between NaCN and TW80 samples. The proteins in NaCN samples began to aggregate, forming large, lumpy fragments (Fig. 5) In contrast, TW80 samples displayed small oil droplets evenly distributed in a close-knit and consistent protein matrix (Fig. 5).

Another notable difference was observed in the prevalence of oil droplets. TW80 samples contained noticeably more oil droplets and a higher extent of fat globule coalescence compared to NaCN samples throughout the digestion process (Fig. 5). These results confirmed that more oil droplets were trapped in the curds of NaCN samples. Most interestingly, at the end of digestion (240 min), a significant quantity of oil droplets was observed in the digesta of UHT-NaCN (Fig. 5, 240 min). This observation appears to correlate with the lipid emptying pattern, where UHT-NaCN exhibited the lowest lipid content among the samples at 240 min.

3.5. CoQ10 release during gastric digestion

CoQ10 was investigated as a representative lipophilic bioactive compound encapsulated in the oil droplets that were incorporated into the milk system. After illustrating how milk treatment and interfacial composition influence gastric digestion behaviour (including emptying and chyme disintegration), the release profile of CoQ10 in the digesta was monitored throughout gastric digestion. At the start of digestion (30 min), all samples exhibited a lower concentration of CoQ10 in the digesta compared to undigested milk (Fig. 6), indicating a degree of CoQ10 encapsulation within the curd matrix. Notably, PAST-NaCN showed a significantly lower concentration than the other samples, as most CoQ10 was retained in the curd.

As digestion progressed, the release profiles of CoQ10 in TW80 and NaCN samples diverged. In TW80 samples, the concentration of CoQ10 decreased as digestion advanced, approaching zero at the end of digestion (Fig. 6). Conversely, in NaCN samples, CoQ10 concentration remained low in the digesta until 3 h (Fig. 6). There was a steep rise in CoQ10 concentration starting at 150 min, followed by a gradual decrease towards the end (Fig. 6). This pattern suggests that CoQ10 was trapped in the curd and not released until later stages of digestion, marked by a sudden breakdown of the curd and subsequent CoQ10 release. Heat treatments on milk appeared not to affect the release profile in TW80 samples but did impact NaCN samples. This was particularly evident in PAST-NaCN, where the sudden release was delayed, resulting in a high CoQ10 concentration detected at the end of digestion (Fig. 6). This difference indicated that an interaction between

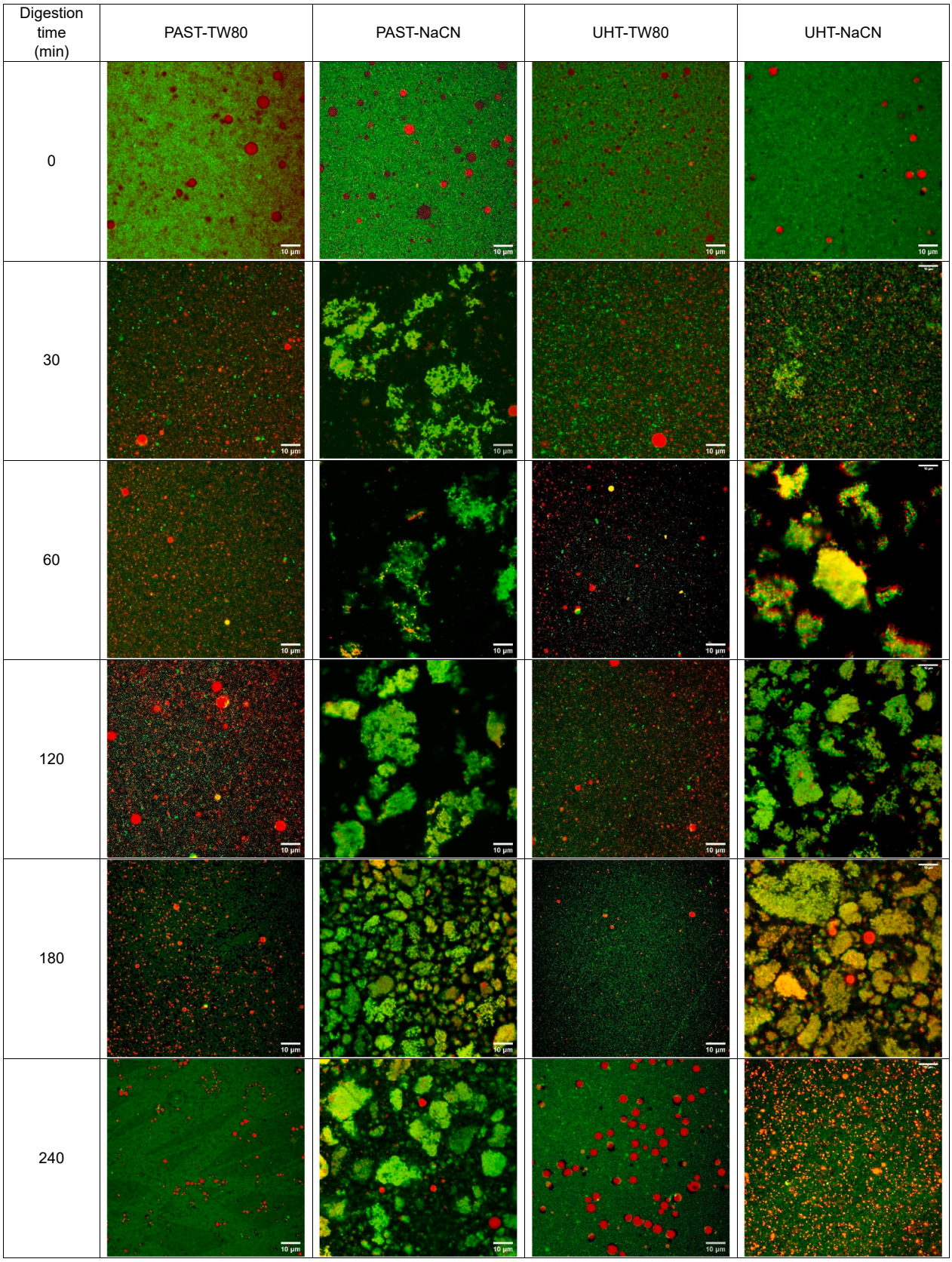

Fig. 5. Microstructure of emptied digesta of Co-enzyme Q10 (Co-Q10) enriched milk at different digestion time detected by confocal laser scanning microscope. Green colour indicates protein while red colour indicates lipid present in the matrices. PAST-TW80: Pasteurized milk fortified with Co-Q10, emulsified using Tween 80. PAST-NaCN: Pasteurized milk fortified with Co-Q10, emulsified using sodium caseinate. UHT-TW80: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using Tween 80. UHT-NaCN: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using sodium caseinate.

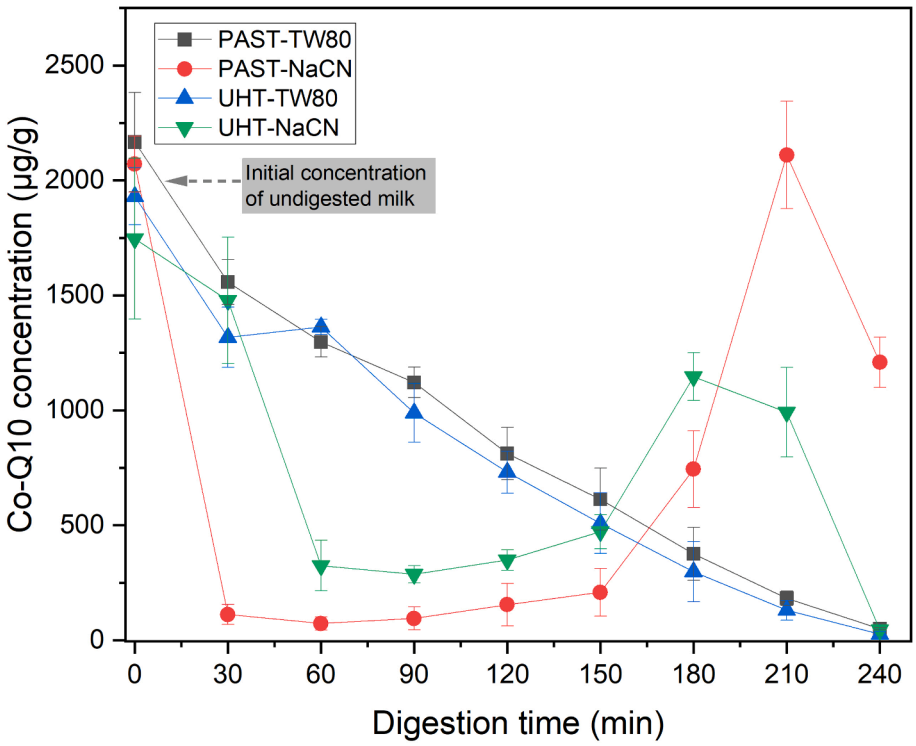

Fig. 6. Change in concentration of Co-enzyme Q10 (Co-Q10) in the emptied digesta of Co-Q10 enriched milk during digestion. PAST-TW80: Pasteurized milk fortified with Co-Q10, emulsified using Tween 80. PAST-NaCN: Pasteurized milk fortified with Co-Q10, emulsified using sodium caseinate. UHT-TW80: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using Tween 80. UHT-NaCN: Ultra-high-temperature (UHT) processed milk fortified with Co-Q10, emulsified using sodium caseinate.

NaCN and the milk structure affects the release of CoQ10.

The retention of CoQ10 in the HGS can be correlated with lipid retention as illustrated in Fig. S3. A linear correlation between CoQ10 and lipid concentrations was observed, exhibiting a very high correlation coefficient (Fig. S3, Pearson's r > 0.8). Consequently, the retention of CoQ10 in the HGS followed the same trend as that of lipid retention.

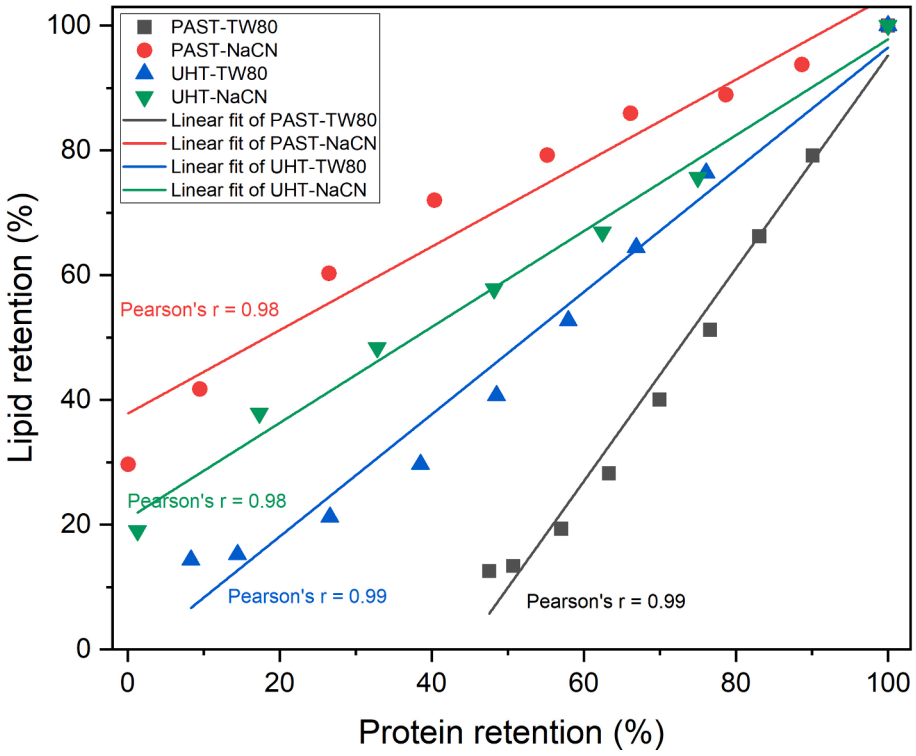

Fig. 7. Correlation analysis of mean values (n = 2 independent *in vitro* digestions) obtained from lipid retention (%) and protein retention (%) in emptied digesta samples of Co-Q10 enriched milk during digestion.

The highest CoQ10 retention in the HGS was observed in PAST-NaCN throughout the digestion process. In contrast, UHT-NaCN demonstrated a slower decrease in CoQ10 concentration at the beginning of digestion, up to 3 h, compared to TW80 samples. Starting at 150 min, CoQ10 in UHT-NaCN was rapidly depleted, reaching the lowest content at the end of digestion among all samples. This suggests that UHT treatment weakened the protein structure, making it more susceptible to breakdown. NaCN's interaction with milk protein accelerated oil coagulation, leading to an extensive release of CoQ10. Qazi, Ye, Acevedo-Fani, and Singh (2021) also demonstrated that the digestion kinetics of protein gels in the stomach affect the availability of oil for lipolysis and the bioaccessibility of fortified lipophilic bioactive compounds in curcumin-enriched dairy protein matrices.

3.6. Relationship among interfacial composition, curd breakdown, and CoQ10 release

The results above suggested that the interactions between CoQ10 emulsion droplets (especially between interacting and non-interacting interfacial agents, NaCN and TW80) and the milk matrix therefore affect milk coagulation, curd breakdown, and the release kinetics of CoQ10. To further analyze the relationship among interfacial composition, curd breakdown, and CoQ10 release, correlation analyses were conducted between between lipid retention and protein retention. Strong positive linear correlations (Pearson correlation coefficient, $r \geq 0.98$ 0.957) was found between the amounts of lipid and protein retained in the stomach throughout gastric digestion (Fig. 7). This indicated that lipid retention was proportional to the protein in the stomach. Oil droplets might be evenly distributed and released when the curd structure was broken down, which was also demonstrated by Ye et al. (2016) and Ye et al. (2017) in studies demonstrating fat release in different whole milk systems. Even though heat treatment and homogenization affect the curd structure and protein hydrolysis, and therefore influence the way fat globules are entrapped in the matrix, the manner in which the fat globules are incorporated within the curd matrix does not control their release. This finding was later also confirmed by Roy et al. (2021) in studies of fat release from whole milk from different species as well as heat treatments during gastric digestion.

However, the CoQ10-enriched milk did not behave the same as the whole milk system due to the incorporation of a recombined colloidal system with a different interfacial composition. Compared with non-interacting TW80 stabilized samples, NaCN samples consistently showed significantly higher lipid retention at equivalent protein levels remaining in the stomach during the entire digestion period (Fig. 7). Unlike MFGM proteins in whole milk, which do not interact with the milk matrix, NaCN can interact with caseins originally in the milk during coagulation and breakdown, building a stronger network to enwrap coalesced oil droplets, resulting in large and stable oil droplets in the matrix. In other words, TW80 does not interact with the milk matrix, nor does it contain protein like MFGM protein at the interface of the oil droplets. Therefore, TW80-stabilized oil droplets are less susceptible to coalescing. As the CoQ10 milk is a recombined milk system containing

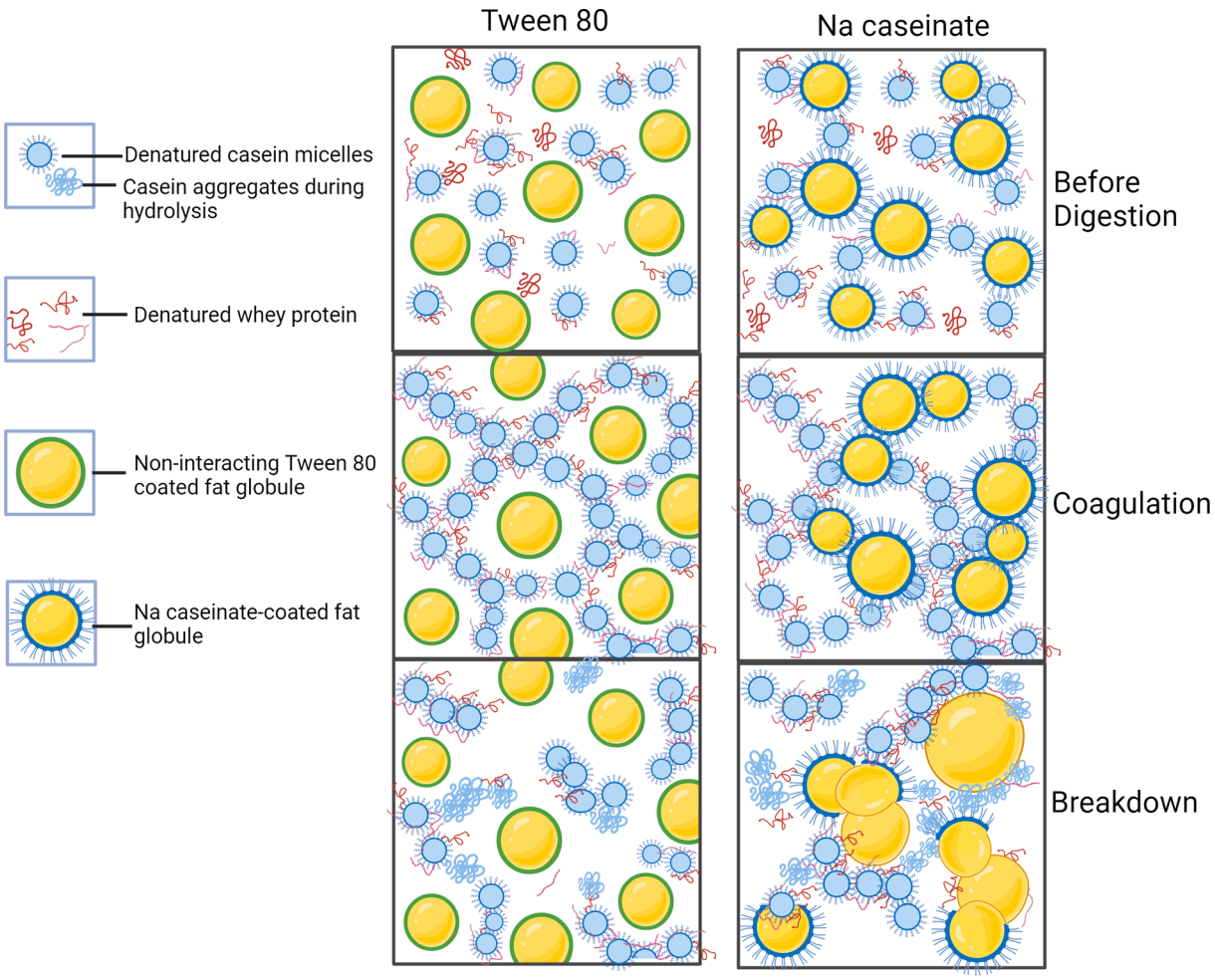

Fig. 8. Graphic illustration of gastric behavior of CoQ10-enriched milk prepared from CoQ10-loaded emulsions stabilized by non-interacting Tween 80 (TW80) and interacting sodium caseinate (NaCN). The illustration depicts the interactions between emulsion droplets and heat-treated (pasteurized or UHT) milk proteins prior to gastric digestion, during coagulation, and through curd breakdown.

different ingredients from whole milk, the protein structure changes caused by heat treatments also affect the release of the oil droplets. Overall, the release of fat or CoQ10 is dependent on the interactions between the interfacial agents and the milk matrix.

The curd structure determines the rate of breakdown and the rate of fat and protein release. This aligns with observations from previously mentioned studies. The soft and loose curd structure showed faster rates of disintegration of the protein matrix and oil droplet release compared with strong and close-knit structures. This was also observed and demonstrated in emulsion-incorporated milk systems (Guo et al., 2016; Qazi, Ye, Acevedo-Fani, & Singh, 2022). Based on the results and discussion in this study, a schematic illustration summarizing the possible interactions between the interfacial composition and the milk matrix under dynamic gastric digestion conditions is provided in Fig. 8.

Before digestion, both CoQ10-enriched milk systems are stable, with intact oil droplets. Due to heating, denatured whey proteins associate with casein micelles to form a stable hydrocolloid liquid system. NaCN-coated oil droplets interact with the surrounding casein micelles and denatured whey proteins, whereas TW80 droplets do not interact with the milk matrix. At the beginning of gastric digestion, milk proteins coagulate due to enzyme hydrolysis. This coagulation leads to the formation of a three-dimensional protein network, primarily built up by casein aggregation. CoQ10 oil droplets are trapped within the protein curd matrix. TW80-coated oil droplets are physically entrapped in the network, while NaCN-coated oil droplets interact with the protein matrix, leading to partial coalescence and a less homogeneous network. As digestion progresses, both curds break down gradually through protein hydrolysis. The 3-D network is disrupted into fragments composed of casein and whey aggregates. TW80 droplets mostly remain intact and are emptied simultaneously with the hydrolysed proteins. The NaCN interface is disrupted by proteolysis and interactions with surrounding proteins, resulting in obvious oil coalescence among the oil droplets. Due to newly formed interactions between protein fragments, the coalesced oil is trapped in the protein network, causing delayed emptying compared with TW80 oil droplets.

4. Conclusion

This study investigated the curd disintegration kinetics and gastric emptying of bovine milk (UHT vs. pasteurized), enriched with coenzyme Q10-loaded emulsions featuring different interfacial compositions (Tween 80 vs. NaCN), and their impact on nutrient release in gastric fluids. Interactions between interfacial composition and milk matrix affect the curd structure, curd disintegration, protein hydrolysis and CoQ10 release. Most notably, an interaction between the active emulsifier NaCN and the milk protein structure led to a fragmented curd structure in PAST-NaCN and a creamy, consistent structure in UHT-NaCN. Additionally, UHT-NaCN exhibited the most rapid curd disintegration and nutrient release. Among the four samples, a strong and rigid protein structure in the stomach, combined with active filler emulsifier encapsulation interacting with the protein matrix, contributed synergistically to the slow release of bioactive compounds. Conversely, an active filler associated with a loose and soft protein structure in the stomach resulted in a faster emptying of bioactive compounds. This insight can contribute to the development of milk-based functional beverages that deliver bioactive compounds with various health benefits. In addition, the mechanisms of interactions between interfacial composition and heat treatments demonstrated in this study can guide the customization of lipophilic bioactive compound delivery to achieve specific release profiles.

CRediT authorship contribution statement

Xinya Wang: Writing – original draft, Visualization, Formal analysis, Data curation. **Peter Zhu:** Investigation. **Aiqian Ye:** Writing – review & editing, Supervision, Methodology. **Harjinder Singh:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Alejandra Acevedo-Fani:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.115774.

Data availability

Data will be made available on request.

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