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Secretion of inflammatory mediators by human intestinal epithelial cells incubated with gastric digesta of emulsion gels containing capsaicinoids: Implication on gastric irritation

Nan Luo [a,b], Frances M. Wolber [b], Harjinder Singh [a], Aiqian Ye [a,b,*]

[a] Riddet Institute, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand
[b] School of Food and Advanced Technology, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand

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ABSTRACT

An *in vitro* method was developed to test gastric irritation by quantifying the secretion of interleukin-8 (IL-8) by human intestinal epithelial cells Caco-2 after incubation with gastric digesta of emulsion gels containing capsaicinoids (CAP) obtained from simulated dynamic gastric digestion. The emulsion gel structure was modified using different emulsifiers: whey proteins versus Tween 80. Results indicate that both the CAP and Tween 80 molecules were proinflammatory to Caco-2 cells and stimulated cells to produce IL-8. Gastric digesta from CAP-loaded Tween 80 emulsion gel stimulated significantly more IL-8 production than CAP-loaded whey protein emulsion gel, possibly because of the presence of Tween 80 and also, because more CAP molecules were released from Tween 80 emulsion gel during gastric digestion. Tween 80 emulsion gel had a loose structure; it was easily broken down into smaller pieces and had large amounts of oil droplet liberation from the protein matrix, which would promote the release of CAP molecules, leading to higher IL-8 production. On the other hand, whey-protein-coated oil droplets had strong connections with surrounding protein matrix and were well protected during gastric digestion; the release of CAP molecules was much less. This study suggests that by modifying the structure of the foods, the gastric digestion behaviour can be modified, which would affect the release behaviour of CAP molecules and influence gastric irritation / inflammation.

1. Introduction

The bioactive compounds capsaicinoids (CAP), present in various peppers from the genus *Capsicum*, are responsible for causing sensory responses such as burning, irritation and pain when they get in contact with the human body. The sensory responses are closely related to their actions on a receptor protein called transient receptor potential vanilloid subtype 1 (TRPV-1) or vanilloid receptor subtype 1 (VR-1) (Frias & Merighi, 2016). TRPV-1 is highly expressed in a subset of sensory neurons which respond to mechanical, thermal and chemical stimuli; there is also evidence showing that TRPV-1 is expressed in non-neuronal cells, such as the epithelial cells of the gastrointestinal tract (Geppetti & Trevisani, 2004; Kato et al., 2003; Ward, Bayguinov, Won, Grundy, & Berthoud, 2003). Upon activation of TRPV-1, the cells release neuropeptides that induce neurogenic inflammation (Richardson & Vasko, 2002). Moreover, TRPV-1 is found to be upregulated in several human pathological conditions, such as gastrointestinal inflammation and ulcerative colitis (Geppetti & Trevisani, 2004; Yiangou et al., 2001). So, the inflammatory/irritation effect of CAP is possibly through the TRPV-1-dependent pathway.

The intestinal epithelium, comprising different cell types such as enterocytes, M cells, goblet cells, immune cells, etc. functions as a permeable barrier that plays an important role in modulating solute and fluid exchange and has direct consequences on nutrient uptake and transportation. The mucosal integrity is challenged daily by external factors in the luminal environment, and the loss of intestinal barrier function can result in clinical and nutritional consequences such as inflammation and malnutrition (Ponce de León-Rodríguez et al., 2019). The human colon epithelial cells *in vitro* provide signals that are essential for initiating and amplifying acute mucosal inflammatory responses (Jung et al., 1995), and the Caco-2 cell line has been widely used to test the effects of food components on intestinal inflammation (Iftikhar et al., 2020; Ponce de León-Rodríguez et al., 2019).

Inflammation is a non-specific, harmonized reaction of the immune

* Corresponding author at: Riddet Institute, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand.
E-mail address: A.M.Ye@massey.ac.nz (A. Ye).

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system, which can be stimulated by various internal (e.g. cell lysis) and external (e.g. microbial) factors (Iftikhar et al., 2020). The initial inflammatory response includes activation of macrophages resident in the gut tissue, which results in the secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor α (TNF-α) or anti-inflammatory factors such as interleukin-10 (IL-10) (Kagnoff, 2014). The pro-inflammatory / anti-inflammatory effects of the food components on intestinal epithelium can be measured by quantifying these cytokines produced by Caco-2 cells.

Emulsion-based systems have been studied for the oral delivery of CAP, where CAP molecules are dissolved in emulsion droplets for the purposes of increased solubility, reduced mouth burn perception, reduced irritation and increased bioaccessibility (Lu et al., 2016; Luo et al., 2019, 2020; Zhu et al., 2015). Lu et al. (2016) studied the gastric mucosa irritation of CAP-loaded nano emulsion and free CAP water suspension by feeding them to rats, and then observing the stained histological tissues of gastric mucosa under the microscope. They found that CAP-loaded nano emulsion alleviated the gastric mucosa irritation as compared to free CAP water suspension. However, limited information is available on *in vitro* methods to test the irritation of CAP-loaded formulations during gastric digestion.

In this study, we aim to establish an *in vitro* method to quantify gastric irritation by measuring the stimulated secretion of the inflammatory mediators IL-8 by Caco-2 cells after incubation with gastric digesta of CAP-loaded emulsion gels obtained from simulated dynamic gastric digestion. The structure of the CAP-loaded emulsion gel was modified using different emulsifiers: whey proteins versus Tween 80, to investigate how food structure and digestion behaviour affect gastric irritation.

2. Materials and methods

2.1. Materials

Powdered CAP (61 % capsaicin, 32 % dihydrocapsaicin and 2.5 % other CAPs) was purchased from Wuxi AccoBio Biotech Inc., Wuxi, Jiangsu, China. Whey protein isolate 895 (WPI), instantized and with 93 % protein content, was purchased from Fonterra Co-operative Group Limited, Auckland, New Zealand. Food-grade polysorbate 80 (or Tween 80) was purchased from Hawkins Watts Ltd, Auckland, New Zealand. Soybean oil was purchased from Davis Trading Company, Palmerston North, New Zealand, and was used without further purification. Milli-Q water (Millipore Corp., Bedford, MA, USA) was used for all experiments. Pepsin from porcine gastric mucosa (#P7000: $\geq$ 250 units/mg solid), pancreatin from porcine pancreas (#P7545: 8 × USP), amano lipase A from *Aspergillus niger* (#534781: $\geq$ 12 000 U g^{-1}), bile bovine (#B3883) and Pefabloc® SC (#76307) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemical reagents used in this study were of analytical grade and were used without further modification, unless otherwise specified.

2.2. Methods

2.2.1. Preparation of CAP-loaded whey protein emulsions

Powdered CAP and WPI were added to soybean oil and water respectively. The solutions were magnetically stirred for 4 h at room temperature to allow complete dissolution. A CAP-loaded whey protein coarse emulsion containing 0.02 wt % CAP, 19.98 wt % soybean oil and 10 wt % WPI was prepared using a high shear mixer (L4RT, Silverson, East Longmeadow, MA, USA) at 8000 rev min^{-1} for 5 min. The coarse emulsion was then homogenized by passing it four times through a two-stage valve homogenizer (Homolab 2, FBF ITALIA SRL, Sala Baganza, Parma, Italy) at first-stage/second-stage pressures of 250/50 bar to generate an average oil droplet size ($d_{4,3}$) of ~ 0.5 ± 0.05 μm with an monomodal distribution Luo et al. (2023).

2.2.2. Preparation of CAP-loaded Tween 80 emulsions

Powdered CAP was added to soybean oil; Tween 80 and WPI were added to water. The solutions were magnetically stirred for 4 h at room temperature to allow complete dissolution. A CAP-loaded Tween 80 stock coarse emulsion containing 0.03 wt % CAP, 29.97 wt % soybean oil and 3 wt % Tween 80 was prepared using a high shear mixer (L4RT) at 8000 rev min^{-1} for 5 min. A CAP-loaded Tween 80 stock emulsion with an average oil droplet size ($d_{4,3}$) of ~ 0.5 ± 0.05 μm with an monomodal distribution Luo et al. (2023) was generated under the same conditions as described in Section 2.2.1. The CAP-loaded Tween 80 stock emulsion was then mixed with stock WPI solution (30 wt % WPI) at a mass ratio of 2:1 to achieve a CAP-loaded Tween 80 emulsion with final concentrations of 0.02 wt % CAP, 19.98 wt % soybean oil, 2 wt % Tween 80 and 10 wt % WPI. The whey proteins were added after emulsification to ensure that there was no protein adsorption at the oil–water interface and that the whey proteins were present as a gelling agent only.

Simulated salivary fluid (SSF) and simulated gastric fluid (SGF) were prepared following the instructions from Minekus et al. (2014) with slight modifications. The 1.25 × concentrates of the simulated digestion fluids are referred to as stock simulated digestion fluids. The stock simulated digestion fluids were magnetically stirred at room temperature for 2 h to allow complete dissolution. The pH values of the stock SSF (1.25 ×) and stock SGF (1.25 ×) and were adjusted to 7.0 and 1.5 respectively using 6 M HCl/10 M NaOH. They were stored at 4 °C, warmed to room temperature in a water bath before use and used within 1 month after preparation.

2.2.3. Formation of CAP-loaded emulsion gels

The required quantities of NaCl were added to the CAP-loaded emulsions to give a final concentration of 100 mM NaCl. They were gently stirred for 1 h to allow complete dissolution of the NaCl. The emulsions were then poured into plastic tubes (inner diameter, 25 mm; capacity, 35 mL), sealed, heated in a water bath from 30 to 90 °C for 10 min and held at 90 °C for 20 min. The plastic tubes were immersed in an ice bath immediately after heating and then stored at 4 °C overnight until further use.

2.2.4. Preparation of simulated masticated gel bolus

A food processer (The Mini Wizz food chopper, BFP100, Breville Group Ltd, Australia) was used to mimic oral breakdown of the CAP-loaded emulsion gels and to produce *in vitro* masticated gel boluses that had similar bolus particle sizes to the *in vivo* masticated gel boluses (Luo et al., 2020). The CAP-loaded emulsion gels were cut into cylinders of 12 mm in height and 25 mm in diameter; then seven cylindrical gel samples were added into the processer. The CAP-loaded whey protein emulsion gel was initially ground for 3 s, and then a portion of ~ 10 g was taken out; the rest was ground for another 5 s, and then ~ 10 g was taken out; the remaining gel particles were ground for another 9 s; all three portions were then mixed together. The CAP-loaded Tween 80 emulsion gel was initially ground for 2 s, and then a portion of ~ 15 g was taken out; the rest was ground for another 2 s, and then ~ 15 g was taken out; the remaining gel particles were ground for another 3 s; all three portions were then mixed together. Experiments were carried out at room temperature. Simulated masticated gel boluses were prepared by mixing 160 g of ground gel with 50 mL of SSF [consisting of 40 mL of stock SSF, 0.5 mL of $CaCl_2$ (0.3 M) and 9.5 mL of water].

2.2.5. Human gastric simulator

A dynamic gastric digestion model—the HGS designed by Kong and Singh (2010)—was used for the *in vitro* gastric digestion. A mesh bag with a pore size of 1 mm was placed inside the latex stomach chamber to mimic human gastric sieving. An enzyme solution containing 1.7 % (w/v) pepsin, 0.275 % (w/v) amano lipase A and 0.54 mmol L^{-1} of $CaCl_2$ was prepared to obtain pepsin and lipase activities of 1000 U mL^{-1} and 50 U mL^{-1} respectively and a calcium ion concentration of 0.075 mmol

L^{-1} in the final gastric digestion mixture. The simulated masticated gel bolus (consisting of 160 g of ground gel and 50 mL of SSF) was warmed at 37 °C in a water bath for 2 min and then added into the latex stomach chamber. A 70 mL aliquot of SGF (consisting of 56 mL of stock SGF and 14 mL of enzyme solution) was also added into the stomach chamber to mimic the condition during the fasting state when the stomach contains a certain amount of gastric juice (Camilleri, 2006). The gastric digestion time was 240 min. The temperature of the HGS was set and maintained at 37 °C by a heater and a thermostat. The gastric secretion rate was set at 2.5 mL min^{-1} (Hoebler et al., 2002). The stock SGF (1.25 ×) and the enzyme solution were added in separately by two pumps; the secretion rates were 2 mL min^{-1} for the stock SGF and 0.5 mL min^{-1} for the enzyme solution. Gastric emptying started after 30 min because of the lag phase of solid foods (Siegel et al., 1988; Urbain et al., 1989). Every 15 min, 45 mL of gastric digesta was emptied from the bottom, corresponding to an emptying rate of 3 mL min^{-1} (Kong and Singh, 2010).

2.2.6. pH measurement

The initial pH was defined as the pH of the simulated masticated gel bolus after mixing with the fasting state SGF. As access into the HGS was prevented by the simulated gastric contractions, the pH in the HGS at different digestion times was represented by the pH of the emptied gastric digesta.

2.2.7. Measurement of solids content of emptied gastric digesta

The emptied gastric digesta collected at 15 min intervals was dried in an oven at 105 °C for 24 h to determine the dry matter content (A). In addition, a control experiment using 160 g of water instead of ground gel was carried out to determine the dry matter content of the simulated digestion fluids (i.e. SSF and SGF) retained in the emptied gastric digesta at different time points (B). The actual dry weight of the gel particles in the digesta emptied at different digestion times was determined by subtracting B from A.

2.2.8. Determination of particle size distribution of emptied gastric digesta

A MasterSizer 2000 (Malvern Instruments Ltd, Malvern, UK) was used to measure the average diameters and the particle size distributions of the gel fragments of the emptied gastric digesta. The refractive index for the gel particles was set at 1.47. The samples were measured immediately after collection. All measurements were conducted at room temperature with three replicates. The weight-to-volume diameter $d_{4,3}$ (μm) was used to denote the average particle size.

2.2.9. Determination of oil droplet size distribution

A MasterSizer 2000 was used to measure the average diameters and the particle size distributions of the oil droplets in the emptied gastric digesta. The refractive index for the oil droplets was set at 1.47. The weight-to-volume diameter $d_{4,3}$ (μm) was used to denote the average oil droplet size. Aliquots of 3 mL of 5 wt % sodium dodecyl sulphate (SDS) solution and 20 μL of β-mercaptoethanol were added to 2 mL of emptied gastric digesta. The mixtures were then shaken overnight in a water bath at 25 °C until complete dissolution. The dissolved mixtures were used for oil droplet size measurements. All measurements were conducted at room temperature with three replicates.

2.2.10. Cell cultures

Caco-2 cells were grown and maintained in MEM, supplemented with 10% (v/v) fetal bovine serum and 1 % (v/v) penicillin streptomycin glutamine (referred to as "growth medium") in a temperature-controlled humidified incubator at 37 °C with 5 % CO_2 (Thermo Scientific Forma® Direct Heat CO_2 Incubator, Thermo Fisher Scientific Inc., USA). The cells were seeded at 2×10^6 cells per 20 mL in Corning® U-shaped cell culture flasks (capacity: 275 mL, 75 cm^2 cell growth area; Fisher Scientific, USA) and the growth medium was changed every 3 days. When the cell monolayer reached 70–90% confluency, cells were harvested with trypsin-EDTA, washed and passaged into new flasks or into assay plates. The cells were used between passage 16 and 40.

2.2.11. In vitro cytotoxicity of CAP in growth medium sample and gastric digesta of CAP-loaded emulsion gels obtained from in vitro gastric digestion

2.2.11.1. Preparation of CAP in growth medium. CAP in growth medium was prepared as a control sample. Powdered CAP was dissolved in growth medium at 50 μg/mL, stored at 4 °C and used within 3 days after preparation.

2.2.11.2. In vitro cytotoxicity test procedure. The *in vitro* cytotoxicity of CAP in growth medium and the *in vitro* gastric digesta from CAP-loaded emulsion gels emptied at different digestion times were determined by measuring the cell viability of Caco-2 cells using MTT assay. The cells were harvested and seeded in 96-well plates with 100 μL per well at a density of 2×10^5 cells / mL. The cells were incubated at 37 °C with 5 % CO_2 for 48 h for the cells to settle, adhere to the bottom and reproduce. After 48 h of incubation of the cells, CAP in growth medium and *in vitro* gastric digesta were transferred into autoclaved 1.5 mL Eppendorf tubes and sterilized under ultraviolet (UV) light for 30 min. Then, the samples were diluted 1:10 in growth medium. 100 μL of the diluted samples / growth medium / Milli-Q water was added to the wells. The growth medium and Milli-Q water were used as negative and positive control respectively. The cells were incubated for another 24 h at 37 °C with 5 % CO_2, then 100 μL was taken out from each well. 10 μL of MTT in phosphate buffer saline (5 mg/mL, sterilized through 0.22 μm syringe filter) was added to each well and incubated for 3.5 h at 37 °C with 5 % CO_2. After 3.5 h incubation, the formation of purple crystals was verified visually under 100× magnification on an optical microscope. Then, 100 μL of 100 % DMSO was added into each well to dissolve the formazan crystals. The plates were analyzed by a microplate reader (ELx808 Ultra Microplate Reader, Bio-Tek Instruments Inc., Winooski, VT, USA) at wavelength of 550 nm. The number of metabolically cells was calculated as a percentage relative to the positive control group, which was normalized to 100 %. The cytotoxicity of CAP-loaded liquid emulsions and gastric digesta emptied at different digestion times was tested on two independent samples in at least triplicate wells for each sample and each dilution.

2.2.12. Quantification of human interleukin 8 (IL-8) production

2.2.12.3. Sample application and incubation on Caco-2 cells. During *in vitro* gastric digestion of CAP-loaded emulsion gels, the gels were gradually disintegrated and digested. The gastric digesta collected at different digestion times were applied on Caco-2 cells to test human IL-8 production by Caco-2 cells. Caco-2 cells were harvested when they reached about 70% confluency and seeded in 96-well plates with 100 μL per well at a density of 2×10^5 cells / mL. The cells were incubated at 37 °C with 5 % CO_2 for 5 days until they became confluent and ready for sample application and incubation. The growth medium was changed on day 3.

During the gastric digestion process, the pH of the gastric digesta gradually decreased. To investigate the effect of the pH of gastric digesta on IL-8 production, the pH of the gastric digesta was either adjusted to 7.0 or left unadjusted. Gastric digesta samples collected from *in vitro* gastric digestion of emulsion gels containing no CAP were also tested as blank controls. The freshly collected gastric digesta samples (pH either adjusted to 7.0 or not; emulsion gels either contained CAP or not) were transferred into autoclaved 1.5 mL Eppendorf tubes and sterilized under UV light for 30 min. Then, the samples were diluted 1:10 in growth medium.

On day 5 of cell culture on the 96-well plates, the cells became confluent. The growth medium was gently taken out and then 200 μL diluted digesta samples / growth medium / interleulin-1β (Recombinant human IL-1 beta protein (active), #ab9617, Abcam, Cambridge, UK;

prepared at 2 ng/mL in growth medium) were added into each well. The growth medium and interleukin-1β (IL-1β) were used as negative and positive control respectively. The cells were then incubated with samples for 24 h at 37 °C with 5 % CO_2.

The quantification of human IL-8 production by Caco-2 cells after 24 h incubation with gastric digesta samples was carried out with digesta samples collected from two independent digestion experiments (duplicate). In each trial, the samples were tested in triplicate.

2.2.12.4. Quantification of human IL-8 production by enzyme-linked immunosorbent assay (Elisa). The enzyme-linked immunosorbent assays (ELISA) were carried out using the human IL-8 antibody pair kit (Catalog #CHC1303, Invitrogen, Thermo Fisher Scientific Inc., USA) following the protocol provided by the manufacturer. The detailed protocol is as follows.

To prepare the ELISA plates, 96-well plates were coated with 100 μL of coating solution (1 μg/mL of coating antibody in coating buffer A) per well. The plates were covered with aluminum foil to avoid light and incubated for 12–18 h at 4 °C. After incubation, the plates were washed once using a microplate washer with the wash buffer provided in the buffer kit (#CNB0011, Invitrogen, Thermo Fisher Scientific Inc., USA). Following wash, the plates were inverted and tapped on absorbent paper forcefully to remove excess liquid. Then, the plates were blocked with 300 μL of assay buffer per well for 1 h at room temperature. The plates were covered with aluminum foil to avoid light. After blocking the plates for 1 h, the liquids were removed by decantation followed by tapping the plates forcefully on absorbent paper.

After gastric digesta sample incubation on Caco-2 cells for 24 h, these plates were centrifuged at 393.75 × g for 1 min (Heraeus® Megafuge® 1.0R, with microplate carrier 75006447), then 100 μL of supernatant was taken from each well and applied on the Elisa plates prepared as previously described. Human IL-8 standard solutions were prepared in assay buffer with a series of concentrations of 0, 12.5, 25, 50, 100, 200, 400 and 800 pg/mL, following the instructions from the manufacturer. 100 μL of standards (in duplicate) were also added to the Elisa plates. Immediately afterwards, 50 μL of the detection antibody solution (0.04 μg/mL of detection antibody in assay buffer) was added into each well. The plates were then incubated for 2 h at room temperature with continual shaking at 700 rpm. Following that, the plates were washed with the wash buffer for 5 times using the plate washer. Then, 100 μL of streptavidin-HRP solution (0.04 % (v/v) streptavidin-HRP in assay buffer) was added to each well and incubated for 30 min at room temperature with continual shaking at 700 rpm. After 30 min, the plates were washed with the wash buffer for 5 times using the plate washer. Then, 100 μL of the TMB substrate was added to each well and incubated for 30 min at room temperature with continual shaking at 700 rpm. After 30 min, 100 μL of stop solution was added to each well, and the plates were analyzed using the microplate reader at wavelengths of 450 nm and 620 nm. The absorbance was measured within 30 min after adding the stop solution. A 4-parameter curve fit was performed by the software to analyze the results.

2.3. Statistical analysis

Each experiment was performed in triplicate using freshly prepared samples. The results are presented as the calculated means and standard deviations. The data were analyzed by one-way analysis of variance using IBM SPSS Statistics 24 software. Means were compared by Tukey tests at $P < 0.05$.

3. Results and discussion

3.1. Physiochemical characteristics of emptied gastric digesta

The *in vitro* dynamic gastric digestions of CAP-loaded whey protein emulsion gel, CAP-loaded Tween 80 emulsion gel, whey protein emulsion gel without CAP and Tween 80 emulsion gel without CAP were performed in a Human Gastric Simulator for 240 min. The simulated gastric fluid (SGF, pH 1.5) was added into the stomach chamber at a rate of 2.5 mL/min. Gastric emptying only started after 30 min because of the existence of the lag phase during gastric digestion of solid foods (Siegel et al., 1988). Starting from 30 min onwards, 45 mL of the gastric content was emptied out in every 15 min to reach a gastric emptying rate of 3 mL/min. Gastric digesta collected at 0 min consisted of the *in vitro* masticated bolus (160 g grinded gel mixed with 50 mL simulated salivary fluid) as well as 70 mL fasting SGF. Gastric digesta collected at 0, 30, 60, 90, 120, 150, 180, 210 and 240 min of digestion were used in this study. Fig. 1 presents the physiochemical properties, including pH, solid content (w/v%), particle size ($d_{4,3}$, μm) and oil droplet size ($d_{4,3}$, μm) of gastric digesta from CAP-loaded whey protein emulsion gel (0.02 wt % CAP; whey proteins as emulsifier, oil droplet size $d_{4,3} \sim 0.5$ μm, 100 mM NaCl), CAP-loaded Tween 80 emulsion gel (0.02 wt% CAP; Tween 80 as emulsifier, oil droplet size $d_{4,3} \sim 0.5$ μm, 100 mM NaCl), whey protein emulsion gel without CAP (whey proteins as emulsifier, oil droplet size $d_{4,3} \sim 0.5$ μm, 100 mM NaCl) and Tween 80 emulsion gel without CAP (Tween 80 as emulsifier, oil droplet size $d_{4,3} \sim 0.5$ μm, 100 mM NaCl), respectively, as a function of digestion time. Emulsion gels without CAP were used as controls.

Within the gel pairings with or without CAP, none of the four parameters measured showed significant differences, indicating that with both whey protein and Tween 80 emulsion gels, the incorporation of CAP in emulsion droplets had no significant effect on pH, solid content, particle size or oil droplet size over time during the digestive process in the model stomach. The pH did not differ significantly between the two gel types (i.e. whey proteins versus Tween 80 as emulsifier of the emulsion gel). Gastric digesta from Tween 80 emulsion gels had higher solid content initially compared to whey protein emulsion gels, but this difference was lost after 180 min of digestion. The average particle size of the gastric digesta from Tween 80 emulsion gels was about half that of the whey protein emulsion gels at 30 min of digestion, mainly due to its smaller masticated bolus particle size entering the stomach; the gap grew progressively greater as digestion progressed, indicating that Tween 80 emulsion gels were disintegrated faster than the whey protein emulsion gels during gastric digestion. Oil droplet size did not change measurably during digestion of whey protein emulsion gels, while it increased at the end by 50 – 100% during digestion of Tween 80 emulsion gels, indicating that oil droplet coalescence was more severe in Tween 80 emulsion gels. More detailed discussion on the different digestion behaviours between CAP-loaded whey protein emulsion gel and CAP-loaded Tween 80 emulsion gel can be found in Luo et al. (2023).

3.2. In vitro cytotoxicity of gastric digesta on Caco-2 cells

The pH of the gastric digesta was either adjusted to 7.0 or left unchanged after emptying from the stomach, in order to study the effect of the pH of gastric digesta on *in vitro* cytotoxicity. Gerloff et al. (2013) studied the effect of *in vitro* gastric and intestinal conditions on particle-induced cytotoxicity and IL-8 production by Caco-2 and reported that the pretreatment under simulated gastrointestinal conditions of particles did not influence cytotoxicity or IL-8 expression.

Gastric digesta was sterilized under ultraviolet (UV) light for 30 min, then diluted in cell complete growth medium with a dilution factor of 10 before application to Caco-2 cells cultured on the plastic surface of 96 well plates. The incubation time was 24 h. MTT assay was performed to determine the *in vitro* cytotoxicity of gastric digesta on Caco-2 cells. Results were expressed as percentage of viable cells in ratio to the negative control (i.e. Caco-2 cells incubated with cell complete growth medium alone).

Fig. 2 presents the results of viability of Caco-2 cells after 24 h incubation with gastric digesta collected at different digestion times from

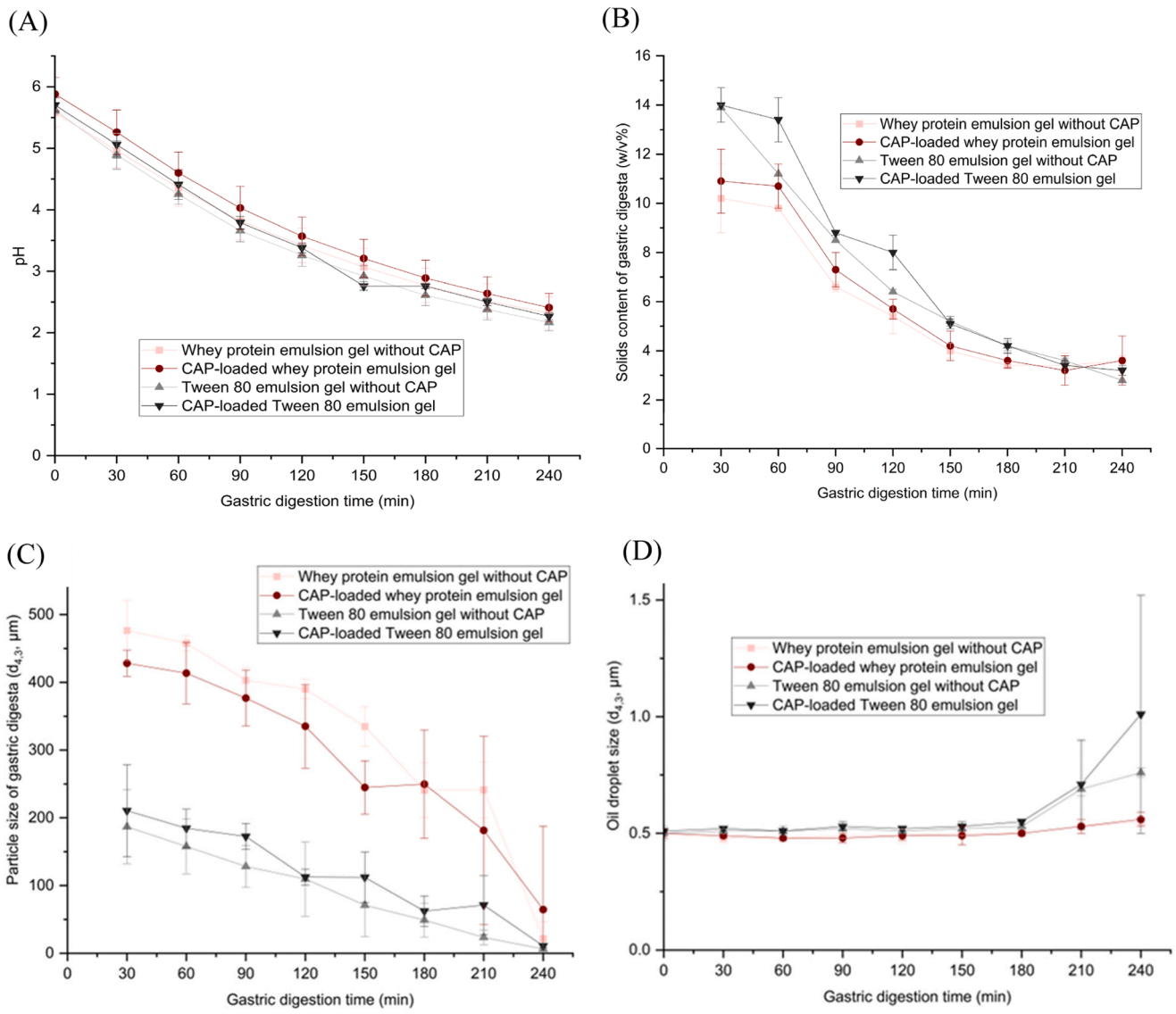

Fig. 1. Physiochemical properties of the whey protein emulsion gel without CAP (no CAP; whey protein as emulsifier, $d_{4,3} \sim 0.5$ μm, 100 mM NaCl), the CAP-loaded whey protein emulsion gel (0.02 wt % CAP; whey protein as emulsifier, $d_{4,3} \sim 0.5$ μm, 100 mM NaCl), the Tween 80 emulsion gel without CAP (no CAP; Tween 80 as emulsifier, $d_{4,3} \sim 0.5$ μm, 100 mM NaCl), and the CAP-loaded Tween 80 emulsion gel (0.02 wt% CAP; Tween 80 as emulsifier, $d_{4,3} \sim 0.5$ μm, 100 mM NaCl) during *in vitro* gastric digestion in the human gastric simulator (HGS): (A) changes in pH; (B) solids content of the emptied gastric digesta as a function of the digestion time; (C) particle size of the emptied gastric digesta as a function of the digestion time; (D) oil droplet size of the emptied gastric digesta as a function of the digestion time. Error bars represent standard deviations obtained from three replicates.

four emulsion gels, where the pH of gastric digesta was left unadjusted. Whey protein emulsion gels with and without CAP showed similar bimodal cytotoxic patterns. The initial digesta at 0 and 30 min of digestion were not cytotoxic. At 60, 90, and 120 min, the digesta resulted in nearly 100 % cytotoxicity. After this, the 150 – 240 min time points returned to having no significant cytotoxicity. This is unlikely to be due to the parameters previously measured (pH, solid content, particle size, or oil droplet size) as none of these showed a bimodal pattern but rather steadily decreased or remained unchanged over time.

There were significant differences in cytotoxic effects only in the final two time points between whey protein emulsion gel without CAP versus with CAP. The gastric digesta from whey protein emulsion gel without CAP collected at 210 and 240 min resulted in slightly more viable cells compared to the CAP-loaded whey protein emulsion gel and this was statistically significant. A possible reason could be that the particle size of gastric digesta decreased drastically at 210 and 240 min of digestion (Fig. 1 C), with the release of free oil droplets from protein matrix (Luo et al., 2023). This in turn may have allowed the CAP molecules to be released into the aqueous phase, where they would have come in contact and stimulated the Caco-2 cells. The cytotoxicity of capsaicin on Caco-2 cells behaves in a dose-dependent manner. Isoda et al. (2001) studied the cytotoxicity of capsaicin on Caco-2 cells by measuring the lactate dehydrogenase (LDH) release after incubation with different concentrations of capsaicin. They found that when capsaicin was present at above 200 μM, the cells released significantly more LDH, indicating its toxicity to the cells. Similar results were reported by Tsukura et al. (2007) where they studied the cytotoxicity of capsaicin on Caco-2 monolayers by cell viability assay using WST-1 reagent and reported that when capsaicin was present at above 200 μM, cell viability decreased significantly; when capsaicin was below 100 μM, it showed no toxicity to Caco-2 cells. We estimated the CAP concentrations presented in each well as a function of digestion time, based on the solid content results presented in Fig. 1 B and the CAP concentration presented to cells from gastric digesta collected at 0 min of digestion was about 40 μM and about 7 μM at 240 min of digestion. The CAP levels were quite low, however, the slight differences between whey protein

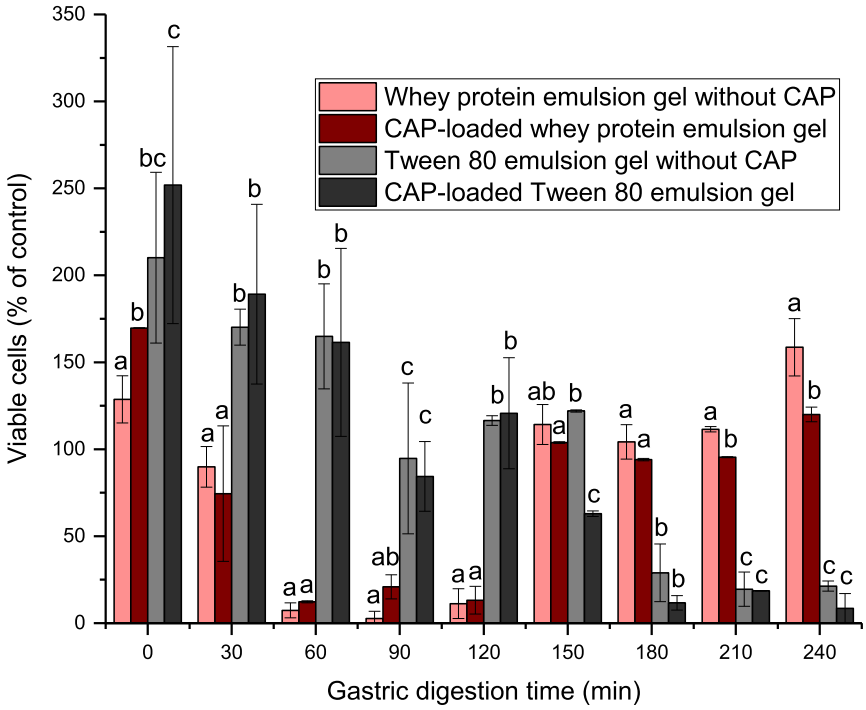

Fig. 2. Viability of Caco-2 cells as determined by MTT assay after 24 h incubation with gastric digesta emptied at different digestion times. pH of gastric digesta was not adjusted. Gastric digesta was diluted 1:10 with cell complete growth medium before application to Caco-2 cells. Error bars represent standard deviations of N = 2 independent experiments, each conducted in duplicate. Different lowercase letters indicate significant differences between samples within the same digestion time point ($P < 0.05$).

emulsion gels with or without CAP seemed to be caused by the presence of the extra CAP molecules, since other factors during digestion were the same (i.e. the digestive enzymes, decrease in pH, etc.).

For gastric digesta from Tween 80 emulsion gels with or without CAP, the cell viability showed a decreasing trend with increasing digestion time. Gastric digesta from Tween 80 emulsion gels with or without CAP did not cause any cytotoxicity from 0 – 120 min of digestion and indeed showed an increase in cell number; however, cytotoxicity/viability is extrapolated from optical density, and it is likely that the gel particles themselves contributed to optical density, causing an artificial increase in the apparent number of viable cells. Even though the results presented here already subtracted the readings from the blank control wells (i.e. wells without cells, incubated with gastric digesta), the gel pieces may have adhered to the cells during incubation and may not have been sufficiently taken out during cytotoxicity measurements. For wells without cells, the gastric digesta was relatively easily and sufficiently taken out, causing the density differences of gel particles between wells with or without cells, leading to the artificial increase in optical density.

Some cytotoxicity was caused by the CAP-loaded Tween 80 emulsion gel at 150 min of digestion. From 180 – 240 min of digestion, both gel types reduced cell viability by approximately 70 %. This pattern is unlikely to be due to pH; although the pH of these digesta also did decrease over time, the whey protein emulsion gels had similar pH levels, and they were not cytotoxic at the later time points. It may be due to the release of Tween 80 into the digestive fluid, as this surfactant has been shown to be cytotoxic to Caco-2 cells at concentrations of 0.1 % to 0.25 % (Lu et al., 2014; Shah et al., 2004). We estimated the concentrations of Tween 80 presented in each well as a function of digestion time, based on the solid content results presented in Fig. 1 B and the approximate Tween 80 concentrations presented to cells from gastric digesta were 0.12 % at 0 min of digestion, 0.05 % at 120 min of digestion and 0.02 % at 240 min of digestion. The cytotoxic effect of Tween 80 to Caco-2 cells was more apparent during 180 to 240 min of digestion, because at these digestion time points, the gel particles were broken down into much smaller pieces, with more oil droplet liberated from the protein matrix. Therefore, the chances of Tween 80 also being released and contacting with Caco-2 cells would have increased and stimulated cell death.

No significant difference was found in cell viability between Tween 80 emulsion gels with and without CAP except for gastric digesta collected at 150 min of digestion. At this time point, the gel without CAP caused no cytotoxicity whereas with its CAP-loaded counterpart caused approximately 50 % cytotoxicity. The gels did not differ significantly at this time point for pH, solid content, or the size of particles or oil droplets. It is possible that, as with the whey protein gel with CAP at the later time points, CAP was released from the CAP-loaded Tween 80 emulsion gel at 150 min of digestion. After this time point, the CAP cytotoxicity may have been masked by the general cytotoxicity caused by the release of Tween 80 from both Tween 80 emulsion gels as described above.

Fig. 3 presents the results of viability of Caco-2 cells after 24 h incubation with gastric digesta, where the pH of gastric digesta was immediately adjusted to 7.0 after emptying from the stomach. Adjusting the pH of gastric digesta to 7.0 would inactivate the digestive enzymes. Also, the neutral pH would be favoured by Caco-2 cells in terms of cell viability.

Comparing Figs. 2 and 3, there were clear differences that could only be attributed to the pH adjustment of gastric digesta. Gastric digesta from CAP-loaded whey protein emulsion gel continued to cause a very high degree of cytotoxicity at the 60 – 120 min time points, and this effect was further carried over to 150 min of digestion. However, whey protein emulsion gel without CAP did not cause significant cytotoxicity at any time point. This finding is as inexplicable as the original finding that these gels were cytotoxic during the middle time points of digestion but not the early and late time points. The Tween 80 emulsion gels again showed a purported increase in viable cells at the 0 – 60 min time points; as above, it can be speculated that this is an artefact of the gel particles themselves contributing to optical density. However, in contrast to the

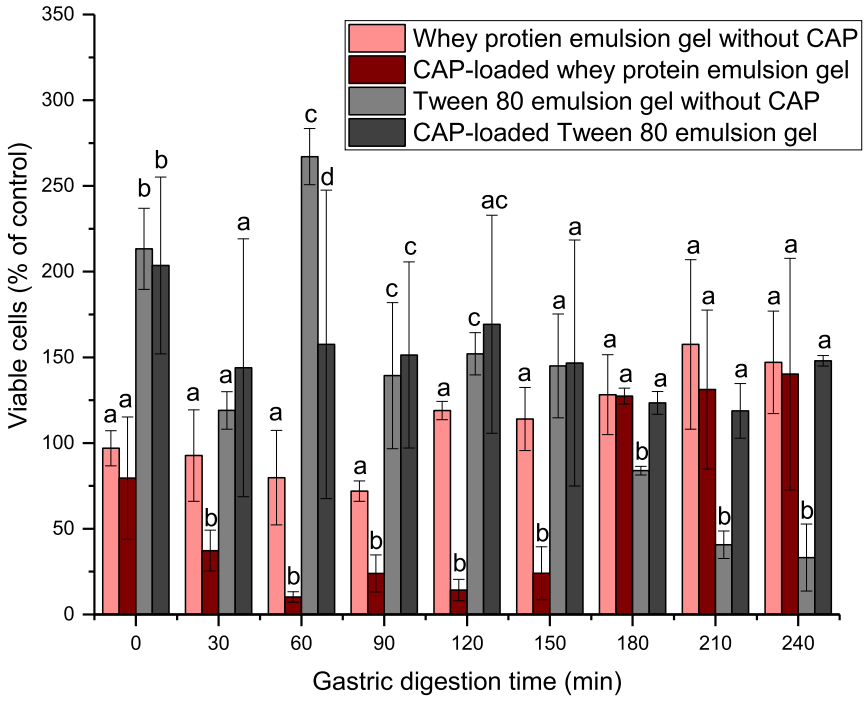

Fig. 3. Viability of Caco-2 cells as determined by MTT assay after 24 h incubation with gastric digesta emptied at different digestion times. pH of gastric digesta was adjusted to 7.0. Gastric digesta was diluted for 10 times with cell complete growth medium before application on Caco-2 cells. Error bars represent standard deviations of N = 2 independent experiments, each conducted in duplicate. Different lowercase letters indicate significant differences between samples within the same digestion time point ($P < 0.05$).

findings with the pH unadjusted samples, Fig. 3 shows that at 150 min there was no difference between Tween 80 emulsion gels with or without CAP; moreover, at the 180 – 240 min the Tween 80 emulsion gel without CAP showed increasing levels of cytotoxicity whereas the CAP-loaded Tween 80 emulsion gel had no cytotoxic effects. This likely rules out the above speculations that in the pH unadjusted digesta, cytotoxicity at the late time points was due to CAP and/or Tween-80 being released into the solution. It is unclear why adjusting the pH altered the cytotoxicity of the whey protein gel without CAP and the Tween 80 gel with CAP, but not their respective counterparts. However, without further exploration of these findings, it is difficult to explain these phenomena.

3.3. *Effect of gastric digesta on human interleukin-8 production by Caco-2 cells*

Fig. 4 shows the production of interleukin-8 (IL-8) by Caco-2 cells after 24 h incubation with diluted gastric digesta samples emptied at different times from whey protein emulsion gel without CAP, CAP-loaded whey protein emulsion gel, Tween 80 emulsion gel without CAP and CAP-loaded Tween 80 emulsion gel, where the pH of gastric digesta was left unadjusted. Gastric digesta was diluted in cell growth medium by a factor of 10, the same concentration was used for the cell viability assay described above. The diluted gastric digesta was used to treat Caco-2 cells for 24 hours. Caco-2 cells were also incubated with 2ng/mL interleukin 1 beta (IL-1β) or growth medium alone as positive and negative controls, respectively. IL-1β is a pro-inflammatory cytokine protein that would stimulate intestinal epithelial cells to produce IL-8 (Fusunyan et al., 1998). IL-8 production by Caco-2 cells after incubation with 2ng/mL IL-1β and complete growth medium was 823.4 ± 279.3 (positive control) and 12.4 ± 6.5 ng/mL (negative control), respectively. The results from positive and negative controls indicate that the Caco-2 cells were responding normally to stimulation and could produce high concentrations of IL-8 within the selected time period. Caco-2 cells were also incubated with a solution of CAP in growth medium at CAP concentration of 50 μg/mL (~ 160 μM). The result of percentage cell viability was 71.4 ± 28.7 %, and the IL-8 production was 75.5 ± 36.7 ng/mL, indicating that CAP had a low level of cytotoxicity to Caco-2 cells and was also able to stimulate Caco-2 cells to produce IL-8.

From Fig. 4 A (i.e. IL-8 production per well), it is apparent that gastric digesta from CAP-loaded whey protein emulsion gel was able to stimulate Caco-2 cells to produce more IL-8 than gastric digesta emptied at the same times from whey protein emulsion gel without CAP, although IL-8 production overall was low. When normalized to the number of viable cells (IL-8 concentration divided by the percentage of viable cells remaining after treatment) in Fig. 4 B, the CAP-loaded whey protein emulsion gel again elicited little to no IL-8 production throughout the digestion period, and the whey protein emulsion gel without CAP induced no IL-8 production. This suggests that the components causing cytotoxicity in these gastric digesta samples, whether active digestive enzymes or aversive pH, do not themselves upregulate IL-8 production, and further that none of the components elicit significant IL-8 even when the cells remain fully viable. Only the presence of CAP molecules appeared to induce any IL-8 production, and this was minimal.

In contrast, gastric digesta from Tween 80 emulsion gels with or without CAP at their natural pH did induce IL-8 production (Fig. 4 B). Gastric digesta from Tween 80 emulsion gel without CAP elicited low levels of IL-8 during the first two hours of digestion even though the cells remained fully viable at these times. At and after 180 min, cell viability was compromised but IL-8 production on a per-cell basis rose to moderate levels. The counterpart gastric digesta from CAP-loaded Tween 80 emulsion gel induced moderate IL-8 production during 0 – 150 min of digestion, when the cells remained fully viable at these times. At and after 180 min, cell viability was also compromised but IL-8 production on a per-cell basis increased to very high concentrations. However, it cannot be determined from these existing data whether the lower number of viable cells in these treatments produced 10-fold more IL-8 on a per-cell basis, or if the full complement of cells produced moderate IL-8

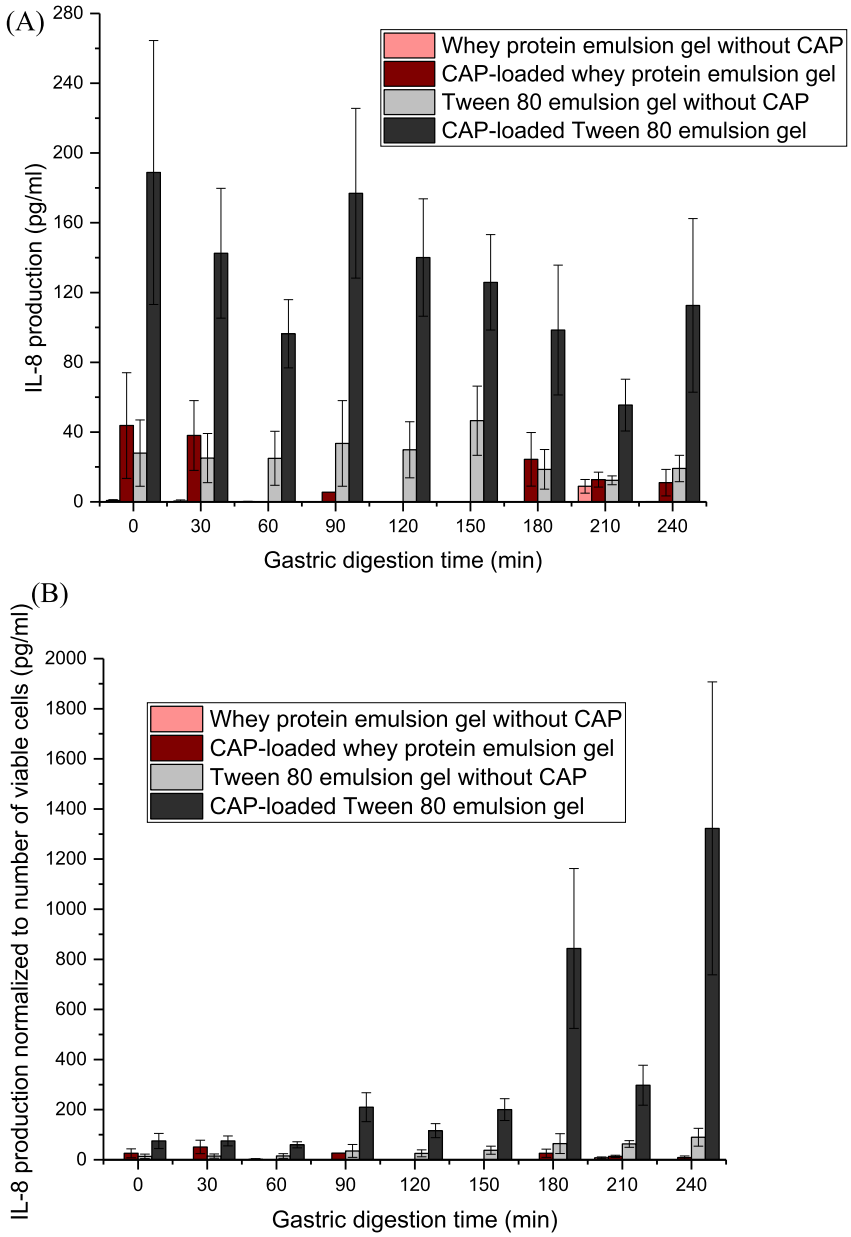

Fig. 4. Effect of gastric digesta collected at different digestion times on IL-8 production by Caco-2 cells. The pH of gastric digesta was left unadjusted. Error bars represent standard deviations of N = 2 independent experiments, each conducted in duplicate. (A) IL-8 production per well; (B) IL-8 production normalized to the number of viable cells.

similar to the earlier time points and subsequently lost viability. The latter explanation is not unlikely given that IL-8 protein in Caco-2 cell conditioned medium peaks at 18 – 24 hours post-stimulation but has been shown to be present in measurable levels within 8 hours of stimulation (Parlesak et al., 2004).

Across the spectrum of assessments, gastric digesta from Tween 80 emulsion gels with unadjusted pH induced more IL-8 production than the matching whey protein emulsion gels, indicating that the matrix did influence inflammation. Moreover, gastric digesta from CAP-loaded Tween 80 emulsion gel stimulated more IL-8 secretion than gastric digesta collected at the same times from CAP-loaded whey protein emulsion gel ($P < 0.05$) and produced the highest overall level of IL-8 on a per-cell basis. This could be the combined effects of the presence of Tween 80 molecules and that there were possibly more CAP molecules released to aqueous phase and exposed to Caco-2 cells in gastric digesta from CAP-loaded Tween 80 emulsion gel. For both types of gel, the inclusion of CAP increased the per-cell production of IL-8, demonstrating that CAP itself induces IL-8; this was particularly apparent for the CAP-loaded Tween 80 emulsion gel. As discussed earlier, Tween 80, as a small-molecule surfactant, is also a proinflammatory factor and toxic substance when presented to Caco-2 cells at sufficient concentrations. It is stated in literature that the exposure of surfactants, such as Tween 20, Tween 60, Tween 85, sucrose esters and etc., to tissues / cells could cause cell damage or cell death and / or trigger the immune response of tissues / cells to secrete inflammation markers such as IL-8 (Lémery et al., 2015; Monteiro-Riviere et al., 2005).

The same assessments were carried out for gastric digesta adjusted to pH 7.0 (Fig. 5 A and B). Fig. 5 A presents the non-normalised IL-8 production by Caco-2 cells after 24 h incubation with gastric digesta, where the pH of gastric digesta was immediately adjusted to 7.0 after emptying from the stomach. Fig. 5 B shows these data normalised to the relative number of viable cells present (as measured and shown in Fig. 3).

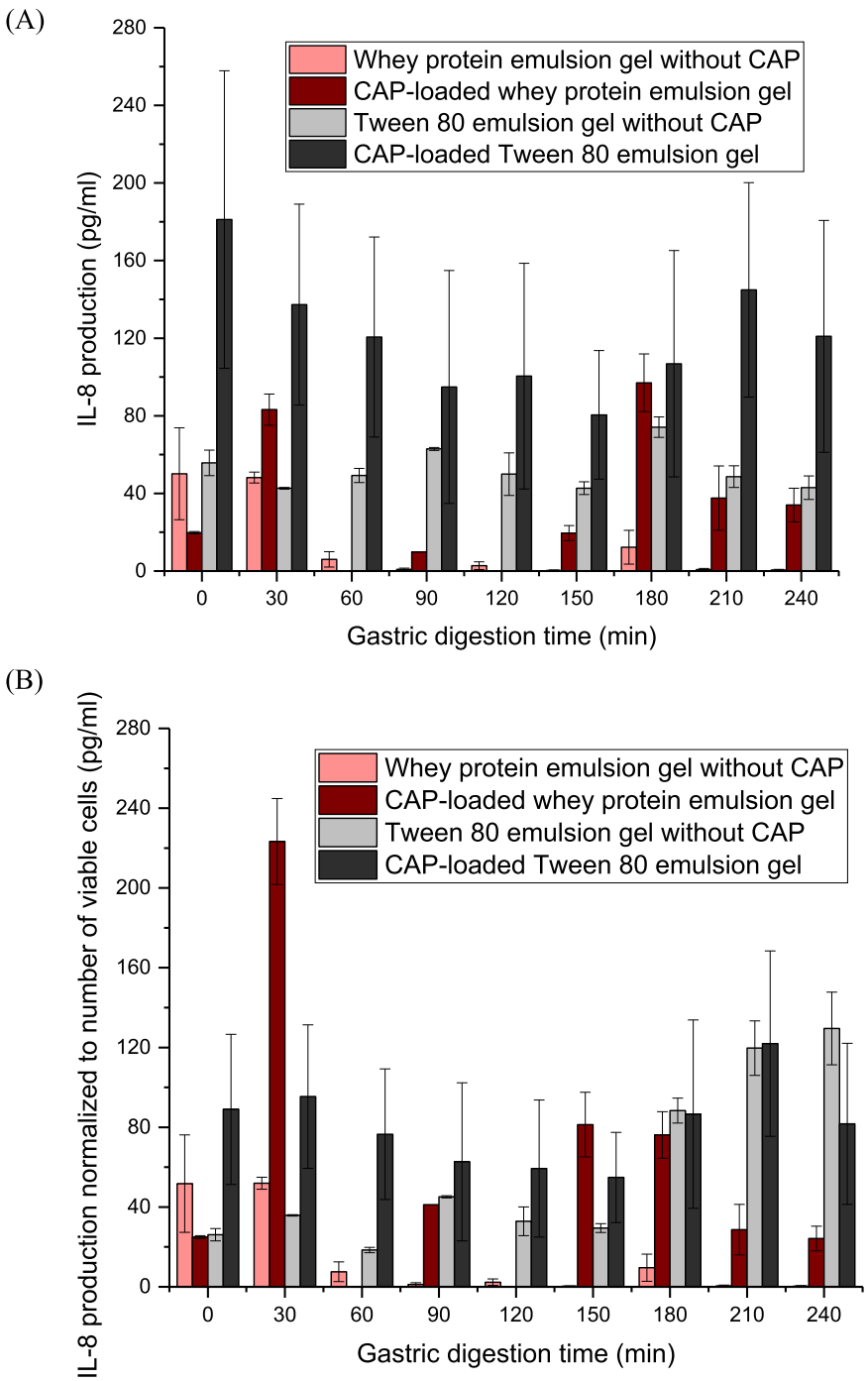

Fig. 5. Effect of gastric digesta emptied at different digestion times on IL-8 production by Caco-2 cells. The pH of gastric digesta was adjusted to 7.0. Error bars represent standard deviations of N = 2 independent experiments, each conducted in duplicate. (A) IL-8 production per well; (B) IL-8 production normalized to the number of viable cells.

In Fig. 5 B, the digesta from whey protein emulsion gels, with or without CAP, induced low to moderate levels of IL-8 in Caco-2 cells on a per-cell basis in a pattern similar to the digesta not adjusted for pH (Fig. 4 B). However, the levels of IL-8 did appear higher when the pH of gastric digesta was adjusted compared to non-adjusted samples, and higher with the CAP-loaded whey protein emulsion gel compared to no CAP.

For gastric digesta from Tween 80 emulsion gels adjusted for pH, normal cell viability had been retained throughout, except for the gastric digesta from Tween 80 emulsion gel without CAP at the final two time points. Fig. 5 A shows that IL-8 production was induced at fairly constant levels throughout the digestion time period. When normalised to viable cell number (Fig. 5 B), the IL-8 production on a per-cell basis was altered only for the 210 and 240 min time points for Tween 80 emulsion gel without CAP. This suggests that IL-8 production was induced in the full complement of cells prior to apoptosis/necrosis being induced, as discussed above. For this reason, the remaining data in this work are presented without being normalized to cell number.

Gastric digesta from Tween 80 emulsion gels with pH adjusted resulted in little cytotoxicity (Fig. 3) yet, though cell numbers were similar to whey protein emulsion gel without CAP, digesta from Tween 80 emulsion gel induced more IL-8 (Fig. 5 A and B). This suggests that

the Tween 80 emulsion gel components can themselves induce IL-8. IL-8 induced from gastric digesta from CAP-loaded Tween 80 emulsion gel was approximately twice as high as that from its counterpart Tween 80 emulsion gel without CAP. Given that the only difference between these two gels is the incorporation of CAP molecules, it can be inferred that CAP was responsible for the differing levels of IL-8 production between the Tween 80 emulsion gels with or without CAP. This would also imply that the same level of CAP was present and able to stimulate the cells starting at time 0 and remaining constant throughout the entire digestive period. However, this is more likely due to differences in the final dilution factor (i.e. dilution of gastric contents due to continuous gastric secretion and emptying), which is discussed in more detail below. A comparison between Figs. 4 B and 5 B shows that IL-8 production was similar for gastric digesta from Tween 80 emulsion gel without CAP regardless of pH adjustment; however, adjusting the pH did lower IL-8 production somewhat with the CAP-loaded Tween 80 emulsion gel.

Since the solid content of gastric digesta gradually decreased with increasing digestion time due to gradual dilution, the IL-8 production was recalculated by multiplying a dilution factor to normalize the solid content of gastric digesta emptied at different times and from different gels. The dilution factors (DF) for gastric digesta at different digestion times from four emulsion gels are calculated based on Equation 1:

$$DF = m\_0/m\_i \quad (1)$$

where, m_0 was the solid content of gastric digesta collected at 0 min of digestion from CAP-loaded Tween 80 emulsion gel. At the beginning of gastric digestion, 160 g grinded CAP-loaded Tween 80 emulsion gel (solid content: 51.2 g) was mixed with 50 mL SSF and 70 mL fasting SGF. The volume of the mixture in the stomach at 0 min of digestion was about 270 mL. Therefore, the calculated theoretical m0 was 19.0 w/t %. Similarly, the calculated theoretical value of solid content of gastric digesta at 0 min of digestion from whey protein emulsion gel with or without CAP was 17.8 w/v % (i.e. 48 g solids in 270 mL mixture). mi was the solid content of gastric digesta emptied at other times from all gels (presented in Fig. 1 B). The results of the dilution factors are presented in Table 1.

The normalized IL-8 production was calculated based on Fig. 4 A and Table 1 and presented in Fig. 6. The pH of gastric digesta was not adjusted to 7.0. Since the results in Fig. 6 were normalized according to the solid content of gastric digesta, the differences between samples came from their differences in composition, gel particle size and colloidal structure of the gastric digesta. Gastric digesta from whey protein emulsion gel without CAP stimulated the lowest IL-8 production among four emulsion gels, while gastric digesta from CAP-loaded Tween 80 emulsion gel stimulated significantly more IL-8 production than gastric digesta collected at the same digestion times from other emulsion gels. The elevated IL-8 production in CAP-loaded whey protein emulsion gel in comparison to the whey protein emulsion gel without CAP came from the presence of the CAP molecules; the effect of CAP molecules on IL-8 production was higher towards the end of gastric digestion, possibly because the particle size of the gastric digesta was smaller, and a proportion of the oil droplets was liberated from the protein matrix, resulting in higher release of CAP molecules from the food matrix. Similar results were observed between Tween 80 emulsion gels with or without CAP. The difference in normalized IL-8 production between Tween 80 emulsion gels with or without CAP was higher than the difference between whey protein emulsion gels with or without CAP. This indicates that there were possibly more CAP molecules been released to the aqueous phase during gastric digestion from CAP-loaded Tween 80 emulsion gel than CAP-loaded whey protein emulsion gel. However, IL-8 production from Tween 80 emulsion gel without CAP was higher than that from either whey protein emulsion gels, suggesting that the Tween 80 itself may induce minor inflammation; this has never been assessed in Caco-2 cells but it has been shown that Tween 80 can induce low levels of IL-8 production directly in keratinocytes (Coquette et al., 1999) and indirectly in blood cells (Kolter et al., 2015).

With increasing digestion time, the normalized IL-8 production showed an increasing trend for CAP-loaded Tween 80 emulsion gel; no such trend was found for other emulsion gels. As discussed previously in Luo et al., (2020) and Luo et al., (2023), CAP-loaded Tween 80 emulsion gel had a loose network structure; the Tween-80-coated oil droplets had no interactions with the whey protein matrix, also, the oil droplets appeared to be in a flocculated state in the protein matrix. During digestion, CAP-loaded Tween 80 emulsion gel was broken down into smaller pieces more easily and released large amounts of oil droplets, as compared to CAP-loaded whey protein emulsion gel. The smaller gel particles, loose network structure and greater release of oil droplets of CAP-loaded Tween 80 emulsion gel led to much higher possibility of CAP molecules being released into the aqueous phase, as compared to CAP-loaded whey protein emulsion gel. Moreover, with increasing digestion time, the gel particles became smaller, and the extent of oil droplet release also increased; therefore, the release of CAP molecules into the aqueous increased with increasing digestion time, leading to the increasing trend in normalized IL-8 production. On the other hand, the oil droplets were mostly kept in the solid gel particles during gastric digestion of CAP-loaded whey protein emulsion gel. Since CAP molecules were dissolved in oil droplets, the extent of oil droplet release would affect the release of CAP molecules into the aqueous phase. Also, larger gel particle size of CAP-loaded whey protein emulsion gel during gastric digestion would also lead to less contact area of the gastric digesta with Caco-2 cells, and therefore, leading to lower inflammatory effect.

Table 1
Dilution factors for gastric digesta at different digestion times from whey protein emulsion gels with or without CAP and Tween 80 emulsion gels with or without CAP. Results are presented as mean ± standard deviation of N = 3.

Gastric digestion time point	Whey protein emulsion gel without CAP	CAP-loaded whey protein emulsion gel	Tween 80 emulsion gel without CAP	CAP-loaded Tween 80 emulsion gel
0	1.07 ± 0.00	1.07 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
30	1.88 ± 0.18	1.59 ± 0.00	1.36 ± 0.01	1.34 ± 0.06
60	1.94 ± 0.00	1.67 ± 0.01	1.69 ± 0.00	1.40 ± 0.08
90	2.85 ± 0.06	2.62 ± 0.07	2.23 ± 0.00	2.15 ± 0.03
120	3.52 ± 0.33	3.62 ± 0.20	2.98 ± 0.04	2.33 ± 0.17
150	4.81 ± 0.28	4.83 ± 0.14	3.67 ± 0.08	3.78 ± 0.08
180	5.58 ± 0.07	5.65 ± 0.52	4.46 ± 0.14	4.64 ± 0.28
210	5.69 ± 0.61	5.76 ± 0.50	5.31 ± 0.12	5.52 ± 0.27
240	5.33 ± 0.26	4.73 ± 0.27	6.83 ± 0.05	6.00 ± 0.38

4. Conclusions

In this study, we investigated gastric irritation using an *in vitro* method by measuring the IL-8 production by Caco-2 cells after incubation with gastric digesta of emulsion gels containing capsaicinoids (CAP) obtained from simulated dynamic gastric digestion. The emulsion gel structure was modified using different emulsifiers: whey proteins versus Tween 80. Emulsion gels without CAP were also used as controls. Results indicate that the presence of CAP molecules in CAP-loaded emulsion gels was able to stimulate Caco-2 cells to produce significantly more IL-8, as compared to emulsion gels without CAP, indicating that CAP molecules are proinflammatory factors to Caco-2 cells and could stimulate the immune response of cells to secrete at least one inflammatory mediator. Gastric digesta from Tween 80 emulsion gel without CAP stimulated more IL-8 production than whey protein emulsion gel without CAP, possibly because of the presence of Tween 80, which is also a proinflammatory factor. Moreover, gastric digesta from CAP-loaded Tween 80 emulsion gel stimulated significantly more IL-8 production than CAP-loaded whey protein emulsion gel. This is the result of the combined effects from the presence of Tween 80 molecules and that there were possibly more CAP molecules released to the aqueous phase from CAP-loaded Tween 80 emulsion gel during gastric digestion. The Tween-80-

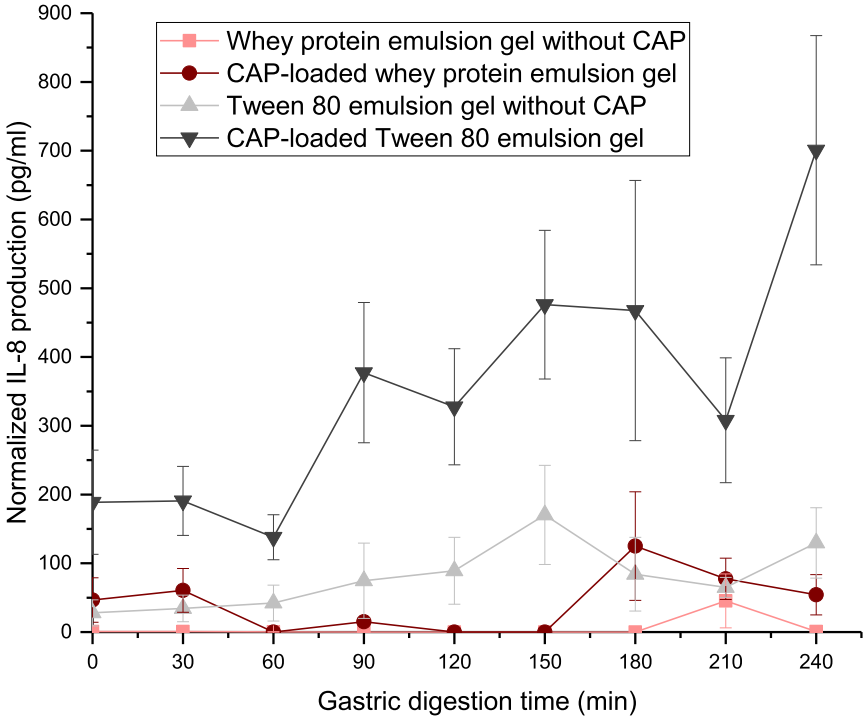

Fig. 6. IL-8 production by Caco-2 cells after 24 h incubation with gastric digesta collected at different digestion times from whey protein emulsion gel without CAP, CAP-loaded whey protein emulsion gel, Tween 80 emulsion gel without CAP and CAP-loaded Tween 80 emulsion gel, normalised by dilution factor to control for digesta content. The pH of gastric digesta was not adjusted to 7.0. Error bars represent standard deviations of N = 2 independent experiments, each conducted in duplicate.

coated oil droplets appeared to be in a flocculated state in the whey protein matrix; they had no interactions with the protein matrix, resulting in a loose gel structure. CAP-loaded Tween 80 emulsion gel was easily broken down into smaller gel pieces and had large amounts of oil droplet liberation from protein matrix throughout gastric digestion, which would promote the release of CAP molecules into the aqueous phase and, therefore, leading to higher IL-8 production. On the other hand, whey-protein-coated oil droplets had strong connections with surrounding protein matrix and were well protected during gastric digestion; the release of CAP molecules would be much less as compared to CAP-loaded Tween 80 emulsion gel. Results demonstrate that by modifying the structure of the foods, the gastric digestion behaviour can be modified, which would then affect the release behaviour of CAP molecule and influence gastric irritation.

Information obtained from this study provides an *in vitro* method for testing gastric irritation / inflammation of CAP-loaded food formulations using Caco-2 cells. Also, this study provides knowledge on designing food structure for the incorporation of bioactive compounds in foods, and providing controlled release of the bioactive compounds. However, the findings presented in this study are preliminary, and it would be of interest to further explore the interactions between CAP, gel structures and its components during digestion on the inflammatory response in gut enterocytes.

Declarations of ethical statement

The authors have declared no studies in humans and animals.

CRediT authorship contribution statement

Nan Luo: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Frances M. Wolber:** Writing – review & editing, Supervision. **Harjinder Singh:** Supervision, Resources, Funding acquisition. **Aiqian Ye:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have declared no conflicts of interest.

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Data availability

Data will be made available on request.

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