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ABSTRACT

The main objective of this study was to assess the ability of the NEar Real Digestive Tract (NERDT), a computer-controlled biomimetic *in vitro* digestion system that considers the biomechanics of the stomach, to reproduce physiologically relevant features of skimmed milk gastric digestion. A second objective was to evaluate the influence of pepsin on the gastric coagulation and emptying of milk proteins from experiments performed with and without pepsin. A mass balance model over the stomach, assuming a perfectly stirred reactor behaviour, has been developed. The results show that the NERDT can adequately reproduce the targeted kinetics of gastric acidification and emptying, with a sieving effect that naturally leads to a delayed emptying of caseins. Milk coagulated earlier and more chyme was emptied towards the end of the experiments in the presence of pepsin than without, hence illustrating the key influence of pepsin on the gastric coagulation of caseins and subsequent hydrolysis and emptying of dairy particles. Overall, this study shows that the NERDT can be adequately controlled to achieve desired gastric digestion conditions, and appears to be a very useful tool to further improve the knowledge of the gastric digestion behaviour of complex foods such as milk.

1. Introduction

Digestion is a complex physiological process that involves several biomechanical and biochemical mechanisms to break down food into nutrients that can be absorbed and used for various metabolic functions. Understanding the fate of food in the gastrointestinal tract is important to deepen our knowledge of the impact of food on human health, for the design of functional foods as well as for the establishment of new dietary recommendations.

Milk is a key source of nutrients for people of all ages, and its behaviour during digestion has been extensively studied. It has been shown that the whey protein fraction, which represents about 20 % of bovine milk proteins, remains soluble and is rapidly emptied from the stomach into the duodenum with limited hydrolysis by pepsin (Boirie et al., 1997). Indeed, whey proteins are known to be quite resistant to pepsin in their native forms, in particular β-lactoglobulin, the main protein of bovine whey (Asselin et al., 1989; Kim et al., 2007). These proteins are also known to be rich in leucine, a branched-chain amino acid that can efficiently stimulate muscle protein synthesis (Rieu et al., 2006). Biologically, the rapid emptying of whey proteins into the small intestine is therefore thought to enable the activation of muscle synthesis metabolism soon after milk ingestion. In contrast, casein micelles, which represent approximately 80 % of bovine milk proteins, coagulate in the stomach because of pepsin hydrolysis of κ-caseins (Tam & Whitaker, 1972; Ye et al., 2016, 2019) and of the acidic conditions in the stomach (Dalgleish & Corredig, 2012). *In vivo* studies with both animal (Miranda & Pelissier, 1981; Roy et al., 2022) and human models (Mahé et al., 1992) all show that the clotting of skimmed milk in the gastric phase leads to a delay in gastric emptying. The coagulation of caseins in the stomach slows down their transit towards the small intestine. Therefore, the constitutive amino acids of caseins are absorbed later and more progressively than the ones contained in whey proteins. This slow arrival of amino acids is thought to enable a prolonged muscle synthesis period after the metabolic pathways has been activated (Boirie et al., 1997).

However, digestion studies performed *in vivo* on humans or animals are not always technically, ethically and financially feasible. They are also associated with poor repeatability due to the high individual variability (Dupont et al., 2019; Guerra et al., 2012). Moreover, *in vivo* studies are of limited interest to deepen our understanding of some key

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mechanisms, such as the respective influences of pepsin action, pH and biomechanics on the gastric digestion behaviour of milk. In the past decades, numerous *in vitro* digestion models have been developed as alternatives to *in vivo* trials. The most advanced dynamic *in vitro* digestion systems include the TNO's gastrointestinal model (TIM) (Minekus et al., 1995), the human gastric simulator (HGS) (Kong & Singh, 2010), the dynamic gastric model (DGM) (Wickham et al., 2012), and the DIDGI system (Ménard et al., 2014). Currently, these models are widely used to study the structural and biochemical changes that occur in different foods or drugs under simulated gastrointestinal conditions (Hur et al., 2011). Compared to the *in vivo* approach, *in vitro* methods are simpler, easier to operate, time-saving, and do not raise ethical concerns (Shani-Levi et al., 2017). However, none of them consider the morphological and anatomical characteristics of the actual stomach, which may hinder the accurate simulation of gastric digestion *in vivo* (Wu & Chen, 2020). In recent years, several digestion models with similar gastric morphology and dimension to the real human stomach have emerged, such as the *in vitro* mechanical gastric system (IMGS) (Barros et al., 2016) and the gastric simulation model (GSM) (Li et al., 2019). These types of gastric models may provide a valuable approach to mimic the process of gastric digestion and emptying as it occurs *in vivo*.

Another biomimetic *in vitro* digestion simulator covering the entire gastrointestinal tract has recently been developed with consideration of the morphological features and anatomical details of the real stomach, including peristalsis. This system, previously named Dynamic *In Vitro* Human Stomach system (DHS-IV), is now commercially available under the name of NERDT, which stands for 'NEar-Real Digestive Tract'. It has been used to investigate the digestive characteristics of several foods such as cheese (Peng et al., 2021), rice (Wang et al., 2022) and yoghurt (Zhang et al., 2023). In these studies, the NERDT was found to adequately simulate many important aspects of digestion, including the gradual decrease in gastric pH, the kinetics of gastric emptying, and the sieving effects on the emptied chyme.

Following on from this work, the objective of the present study was to evaluate the ability of this digestion system to reproduce the gastric digestion behaviour of milk *in vivo*, particularly with regard to the rapid emptying of whey proteins and the delayed emptying of caseins. The impact of pepsin on the gastric digestive behaviour of skimmed milk using NERDT was also investigated in a complementary series of experiments performed without the addition of pepsin.

2. Material and methods

2.1. Chemicals and reagents

A pasteurized skimmed milk powder (SMP) containing 33.8 % of protein, 0.1 % fat, and 55 % lactose (w/w, dry basis) was kindly provided by Eurial, France. Porcine pepsin (P6887-5G), Pepstatin A (P5318-25MG) and *ortho*-phtalaldehyde (P0657-5G) were all bought from Sigma-Aldrich. The pepsin activity was determined to be 4395 U/mg using the pepsin enzymatic assay described in the supplementary material of Minekus et al. (2014). Pepstatin A was dissolved in ethanol and stored at 4 °C before use. Water was Milli-Q water and all other chemicals, unless otherwise stated, were purchased from Sigma-Aldrich and were of standard analytical grade.

2.2. Dynamic in vitro gastric digestion

2.2.1. Experimental devices

The *in vitro* gastric digestion experiments were performed using the NERDT shown in Fig. 1. It is worth noting that gastric emptying is not controlled by a pump in this digestion system. It has been designed to ensure that gastric emptying of food particles is naturally slower than that of liquid food (*i.e.* not directly controlled), via various parameters such as a pyloric opening synchronized with the gastric contractions provided by rollers, and a greater or lesser pyloric opening to retain

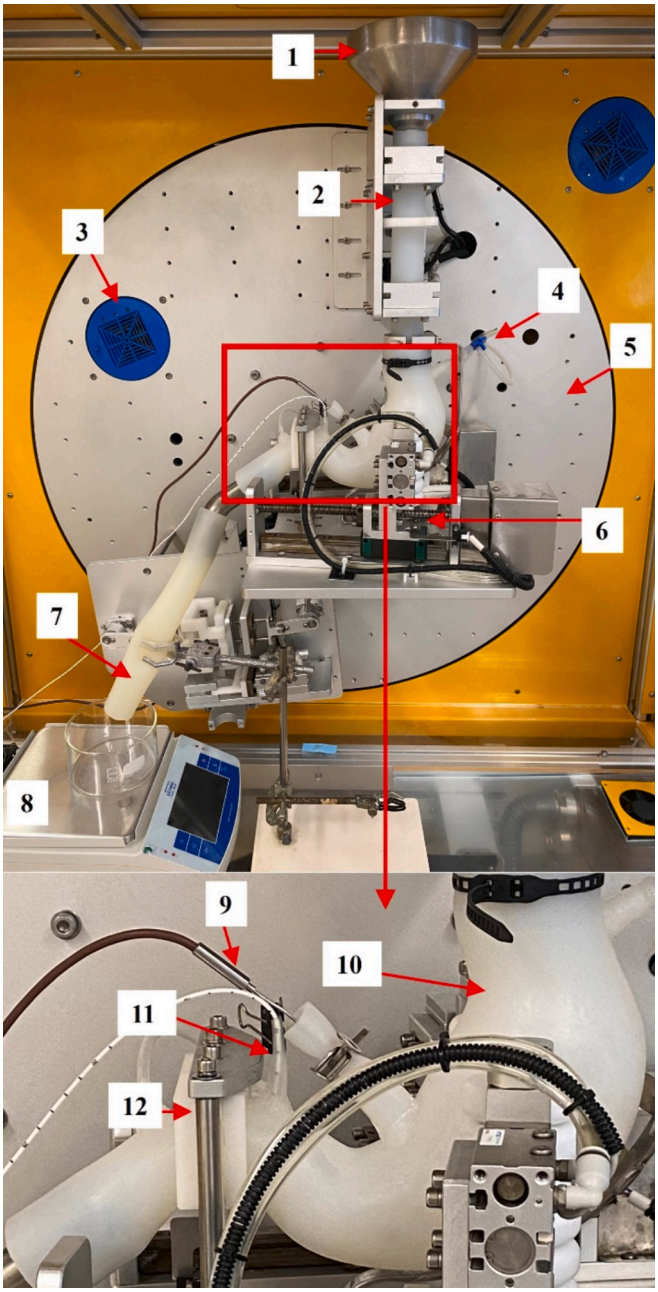

Fig. 1. Picture of the 'NEar Real Digestive Tract' dynamic *in vitro* digestion system (NERDT). 1: funnel for the feeding; 2: esophageal model; 3: heating system; 4: gastric secretion tube; 5: turntable to help controlling the emptying; 6: stomach rolling-extrusion device; 7: tube for chyme collection; 8: scale; 9: thermocouple; 10: human stomach model; 11: soft pH probes; 12: pylorus valve. The scale (8), the thermocouple (9) and the soft pH probes (11) were all connected to a computer to automatically record the mass of emptied material, the intragastric temperature and pH, respectively.

denser and/or larger particles. Similarly, the pH is not regulated either. It results from a whole set of protocol parameters that are applied, which notably include some step-wise procedures of syringe flow rates and of variation rates of the turntable tilting angle. This operating logic implies some difficulties when willing to set the operating parameters of the NERDT to reproduce *in vivo* data. The following paragraphs describe how we proceeded. In addition, a balance (PS 6100.X2, Radwag Balance, Poland) connected to a computer was used to automatically record the mass of the material emptied from the stomach. A thermocouple (753–652, TC Direct, France) and a couple of soft pH probes (Ohmega,

MMS, The Netherlands), placed inside the stomach, were also used to continuously monitor the gastric temperature and pH, respectively.

2.2.2. Target values based on in vivo observations

The *in vivo* situation that we wanted to reproduce *in vitro* corresponds to the gastric digestion of 400 mL skimmed milk in healthy human adults, as studied by Mahé et al. (1992). In order to define the operating parameters of the NERDT, we therefore started by defining some target kinetics or values to be achieved for gastric emptying, gastric pH, and pepsin activity, as follows:

– the target kinetics of gastric emptying was based on the results of Mahé et al. (1992), who reported that the emptying of the liquid phase of the gastric content can be approximated by an exponential decay with a half-gastric emptying time ($t_{1/2}$) of 25 ± 7 min. These considerations were used to compute a target kinetics for the gastric volume (V, mL) using:

$$V = V_{fed} \cdot e^{-k.t} \quad (1)$$

$$k = \left(-\frac{1}{t_{1/2}} \cdot \ln(1 - 0.5) \right) \quad (2)$$

- with V_{fed} (mL) the volume of gastric content after feeding, and with $t_{1/2} = 25$ min.
- the gastric pH was not monitored by Mahé et al. (1992). For this reason, we relied on the results obtained by Malagelada et al. (1979) with an ordinary homogenized meal in humans to define targeted values of gastric pH: pH 4.0 at about 0.5 × $t_{1/2}$ and pH 2.0 at about $t_{1/2}$.
- for pepsin addition, the target value was a pepsin activity of around 2000 U/mL after 2 $t_{1/2}$, an assumption that was based on both the INFOGEST recommendations (Brodkorb et al., 2019) and the results obtained by Nau et al. (2022) with pigs fed egg white gels.

It is noteworthy that the times associated to the target pH and pepsin activity depend on $t_{1/2}$. Although this assumption may not be entirely reliable, it was considered convenient to put the results obtained across different studies with different meal compositions and sizes on a common scale. The setting of the NERDT parameters enabling to fairly reproduce these targeted values or kinetics, and which are provided in the following paragraphs, was thereafter obtained after some preliminary calculations (section 2.3) and experiments.

2.2.3. Experimental protocol and setting of NERDT parameters

For each digestion experiment, 200 mL of reconstituted skimmed milk, obtained from SMP rehydration into osmosis water to achieve a 10 % dry matter content (w/w), was used. As classically done in *in vitro* studies, the volume of the meal used was scaled down compared to the *in vivo* situation (*i.e.* using a scaling factor of 2 in the present study) in order to fill the stomach model of the NERDT to ~ 80 % of its maximum capacity (~300 mL) after accounting for the volumes of basal gastric secretions and Simulated Saliva Fluid (SSF). Assuming an *in vivo* basal gastric fluid content of 24 mL (Maltby et al., 1986; Lydon et al., 1999), 12 mL of basal gastric secretions, adjusted at pH 2, was added to the model stomach to mimic the fasted state. Just before starting an experiment, the 200 mL of skimmed milk was mixed at room temperature with 19 mL of SSF without salivary amylase (37°C). The mixture was then introduced into the digestion system through the cone-shaped funnel within 2 min. The driving device was programmed to produce 3 gastric contractions per minute by moving the rollers in order to reproduce the mean frequency of gastric contractions observed in humans (Quigley, 1996; Schulze, 2006). The pylorus was programmed to open at the end of each gastric contraction using an opening size of 2.5 mm to avoid emptying of food particles larger than ~ 2 mm (Schulze, 2006; Schwizer et al., 2006). Two independent syringe pumps were used for a controlled addition of the simulated pepsin fluid, on the one hand, and of the simulated acidic fluid, on the other hand. Both solutions were prepared using the electrolyte concentrations recommended in the INFOGEST protocol (Brodkorb et al., 2019). In their way to the gastric model, the tubes containing those solutions were heated to 37 °C in a water bath and mixed together via a T-shaped tubbing connection just before they reach the stomach model. The final mixture, corresponding to the Simulated Gastric Fluid (SGF), had an HCl concentration of 0.16 M (Leonard R et al., 2003) and a pepsin activity of 4390 U/mL. Since the pepsin activity in the gastric contents depends on several parameters, such as the amount and activity of the added SGF and the kinetics of emptying, the pepsin activity to be used in the SGF was estimated by modelling using the same approach as the one described in section 2.3. The applied SGF flow rate was maximal at 3 mL/min after the feeding phase, and was gradually decreased down to 0.3 mL/min using a stepwise procedure consisting of 6 steps. The tilting angle of the stomach model was also varied using a stepwise procedure consisting of 6 steps, in a similar manner as in the study of (Wang et al., 2019). The flow rates of the syringe pumps and the tilting angle rates are all provided in Table S1 of the Supplementary Material.

Both the digesta emptied from the stomach and the chyme inside the stomach model were sampled after 5, 14, 20, 27, 40, 52, and 77 min of gastric digestion. These times were chosen so that they correspond to multiples (0.125, 0.5, 1, 1.5, 2, and 3 ×) of the target $t_{1/2}$ of 25 min (Mahé et al., 1992) added with the 2 min of feeding duration. Some samples were collected for immediate microscopic observations. Others were frozen after the addition of Pepstatin A (0.5 mg/mL) for the measurement of protein hydrolysis and dry matter content. All experiments were performed in triplicate.

2.3. Mathematical modelling

To help in the interpretation of the experimental data, a mathematical model that computes mass balances over the stomach model was developed. It was used to predict the dynamic evolution of the dry matter percentages as well as the casein and whey protein concentrations of the digesta emptied from the stomach under the hypothesis that the stomach model behaves as a perfectly stirred reactor (PSR). Under this assumption, the dynamic evolution of the gastric content volume, V (mL), can be described as a function of time:

$$\frac{dV(t)}{dt} = \varnothing_{in}^{F} + \varnothing_{in}^{S} - \varnothing_{out} \quad (3)$$

where $\varnothing_{in}^{F}$, is the feeding flux for $t < t_{feed}$ (the feeding duration) and 0 mL/min for $t > t_{feed}$. $\varnothing_{in}^{S}$ (mL/min) is the flux of incoming secretions, that was controlled by the syringe pump rates, and $\varnothing_{out}$ (mL/min) is the flux of material exiting the stomach.

After feeding, the gastric volume was expected to follow the targeted kinetics previously described (Eq. (1), which relies on the results of Mahé et al. (1992). For $t > t_{feed}$, it can thus be written that:

$$\frac{d(V_{fed} \cdot e^{-k \cdot t})}{dt} = \varnothing_{in}^{S} - \varnothing_{out} \quad (4)$$

which leads to

$$-V_{fed} \cdot k \cdot e^{-k \cdot t} = \varnothing_{in}^{S} - \varnothing_{out} \quad (5)$$

or

$$\varnothing_{out} = V_{fed} \cdot k \cdot e^{-k \cdot t} + \varnothing_{in}^{S} \quad (6)$$

where V_{fed} (mL), the gastric volume after feeding, was estimated to be 234 mL in our experimental conditions. The predicted cumulative mass of emptied material (m_{out}, g), as measured with the scale at the stomach exit, was calculated as:

$$m_{out} = \rho \cdot \sum_{i=2}^{n} \varnothing_{out} \cdot \Delta t_i = \rho \cdot \left(\sum_{i=2}^{n} V_{fed} \cdot k \cdot e^{-k \cdot t} \cdot \Delta t_i + \sum_{i=2}^{n} \varnothing_{in}^{S} \cdot \Delta t_i \right) \quad (7)$$

with ρ the density of the material emptied from the stomach, taken as 1 g/mL.

After feeding, it can also be approximated under the PSR hypothesis that:

$$\frac{dm_{WP}(t)}{dt} = -\varnothing_{out} \cdot \frac{m_{WP}(t)}{V} \quad (8)$$

$$\frac{dm_{Cas}(t)}{dt} = -\varnothing_{out} \cdot \frac{m_{Cas}(t)}{V} \quad (9)$$

which may be transformed into:

$$m_{WP}(t+dt) = m_{WP}(t) - \varnothing_{out} \cdot \frac{m_{WP}(t)}{V} \cdot dt \quad (10)$$

$$m_{Cas}(t+dt) = m_{Cas}(t) - \varnothing_{out} \cdot \frac{m_{Cas}(t)}{V} \cdot dt \quad (11)$$

where m_{WP} and m_{Cas} (g) are the masses of whey proteins and of caseins within the stomach, respectively.

Eq. (10) and (11) were used to predict the dynamic evolution of the whey protein and casein masses in the stomach. These masses were then converted into concentrations of whey proteins (C_{WP}, g/L) and caseins (C_{Cas}, g/L) in the stomach according to:

$$C_{WP}(t) = 1000 \cdot \frac{m_{WP}}{V} \quad (12)$$

$$C_{Cas}(t) = 1000 \cdot \frac{m_{Cas}}{V} \quad (13)$$

with V (mL) the targeted gastric volume at corresponding times (Eq. (1).

Under the PSF assumption, the concentrations at the stomach exit are the same as those in the stomach. These simulations therefore also correspond to the predicted concentrations of whey proteins and caseins in the emptied digesta.

To compute the predicted dry mass percentages (DM, %) at the stomach exit, a similar set of equations were used to predict the gastric concentration of the other milk components (C_{OMC}, g/L). The evolution of the dry mass coming from the secretions (SGF) was also taken into account using:

$$\frac{dm_{DM}^{S}(t)}{dt} = \varnothing_{in}^{S} \cdot C_{DM}^{S} - \varnothing_{out} \cdot \frac{m_{DM}^{S}(t)}{V} \cdot dt \quad (14)$$

$$m_{DM}^{S}(t+dt) = m_{DM}^{S}(t) + \varnothing_{in}^{S} \cdot C_{DM}^{S} - \varnothing_{out} \cdot \frac{m_{DM}^{S}(t)}{V} \cdot dt \quad (15)$$

where m_{DM}^{S} (g) is the gastric mass of dry matter coming from the secretions and C_{DM}^{S} is the corresponding dry matter concentration (estimated to be 0.018 g/mL in our experimental conditions). The gastric concentration of the dry matter coming from the secretions (C_{DM}^{S}, g/L) was then computed as in Eq. (12) and (13). The predicted dry mass percentages of the digesta collected at the stomach exit (DM, %) was thereafter calculated as:

$$DM(t) = 100 \cdot (C_{WP} + C_{Cas} + C_{OMC} + C_{DM}^{S})/(1000.\rho) \quad (16)$$

with the multiplication by 100 to express DM in %, the division by 1000 to convert the concentrations in g/L into kg/L. An alternative simulation was computed by setting C_{Cas} to zero in Eq. (16) in order to predict the dry mass content of the emptied digesta in the case where casein particles are never emptied from the stomach.

2.4. Dry matter content of the samples collected at the stomach exit

An Halogen Moisture Analyzer HE73 (Mettler Toledo SAS, Versailles, France) was used to measure the dry matter content of the digesta collected at the stomach exit. For each measurement, about 1 g of the sample was put onto a filter of the moisture analyzer.

2.5. Optical microscopy

Optical microscopy images of the samples collected from the inside of the stomach and at the stomach exit were acquired using a light microscope (BX51, Olympus, Japan) in a bright field mode with a 10 × magnification. These acquisitions were performed within 10 min maximum after sample collection. Immediately after collecting a sample, it was gently mixed manually, and a 3 mL plastic pipette was used to place a sample drop on a glass slide that was thereafter covered with a coverslip. Several image acquisitions were then performed in different sample locations. One image per time point, considered as representative of the set of images, was later selected in order to be included in the present paper.

2.6. OPA analysis

The concentration of free NH_2 groups in the samples collected at the stomach exit was measured by the OPA method using the protocol described in Lorieau et al. (2018), which relies on the method of Church et al. (1983) adapted to microplates. Briefly, one volume of sample was diluted with three volumes of sodium tetraborate buffer (50 mM). Then, 10 µL of the diluted sample were added to each well and 200 µL of the OPA reagent were added. The mixtures were incubated for 10 min at 37 °C, and the absorbance at 340 nm was measured using a microplate reader (Thermo Fisher Scientific Oy, 1510, Finland) and converted into free NH_2 concentrations using a calibration curve made of L-methionine standard solutions.

2.7. 4th-derivative UV analysis

The 4th-derivative UV method proposed by (Lüthi-Peng & Puhan, 1999) was used to determine the casein and whey protein concentrations in the samples collected at the stomach exit. It should be emphasised that this method is not sensitive to protein hydrolysis, as it relies on the composition of aromatic amino acid residues contained in both kinds of proteins. Therefore, the concentrations measured with that method, further referred to as 'casein' and 'whey protein' concentrations for the sake of simplicity, actually stand for the concentrations of both proteins and peptides coming from caseins and whey proteins. Briefly, a buffer solution containing 6 M guanidine-HCl and 0.1 M sodium acetate was prepared and the pH was adjusted to 5.0. The samples were diluted 100-fold with the buffer solution and vortexed, before measuring their UV spectra from 260 nm to 320 nm using a UV spectrophotometer (SPECTR 0021, SAFAS Monaco) and 1 cm quartz cuvettes. The 4th-derivative spectra were computed using the spectrometer software (SP2000V7, SAFAS Monaco). The total protein concentration and the whey protein to casein ratio of each sample were then estimated from the characteristic peaks of the 4th-derivative spectra (around 284 and 294 nm) and pre-established calibration curves prepared from whey protein isolate and native phosphocaseinate powder. The concentrations of whey proteins and caseins were then computed.

2.8. SDS-PAGE

SDS–PAGE was performed on the samples collected at the stomach exit using 4–12 % polyacrylamide NuPAGE Novex Bis–Tris 15-well precast gels (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Mark 12 Unstained Standard (Invitrogen, Carlsbad, CA) was used as a molecular weight marker. The samples, together with

rehydrated milk, were loaded on the gels at a dilution factor of 40. The bands were also compared to porcine pepsin (P6887-5G, Sigma-Aldrich) solution prepared at 0.5 mg/mL. The gels were stained with colloidal Coomassie Blue (# 1610787, Bio-RAD, USA) and scanned using Image Scanner II (Amersham Biosciences).

2.9. Statistical analysis

Statistical analyses were conducted using R software (version 4.3.0). Unpaired Student's *t*-test at each sampling time were used to compare the effect of the conditions with and without pepsin on: the dry matter percentages, the concentrations of caseins and whey proteins measured by 4th-derivative UV, the concentrations of caseins and β-lactoglobulin measured by SDS-PAGE, and the concentrations in free NH_2. Unpaired Student's *t*-test were also used to determine whether the casein concentrations measured at 52 min and 77 min were different. Differences were considered as statistically significant when p-value was strictly below 0.05. Tendencies are also reported for p-values within the range 0.05–0.10.

3. Results

3.1. Gastric emptying

Fig. 2 shows the evolution of the cumulative mass of emptied material (A), of the gastric volume (B), and the visual appearance of the digesta remaining in the stomach at the end of the experiments (C). The increase in the mass of emptied material was close to the predictions of the mathematical model. By subtracting these results from the cumulative mass of material introduced into the stomach, the volume of the gastric content could be estimated. These results (Fig. 2B) show that the intragastric volume increased rapidly during 2 min of feeding, and then decreased in a trend similar to the targeted exponential decay (Eq. (1). The experimentally observed $t_{1/2}$ was 25.3 ± 2.1 min and 22.3 ± 0.6 min for the conditions with and without pepsin, respectively. These results are also very close to the target value of 27 min (*i.e.* $t_{1/2} + t_{feed}$). The mass of chyme remaining in the stomach at the end of the digestion experiment (t = 77 min) was 23.3 ± 2.0 g and 27.3 ± 1.9 g for the conditions with and without pepsin (p = 0.06), respectively. As shown in Fig. 2C, there was no clear difference in the visual appearance of the final digesta between the two conditions.

3.2. Gastric pH

Fig. 3 presents the pH profiles measured in the conditions with and without pepsin. The very first pH values, at around 2, correspond to the pH of the basal secretions. After feeding, the pH increased rapidly up to ~6.4, close to the pH of skimmed milk, and then gradually decreased in a bell-shaped trend. The pH reached 4 after ~18 min and 2 after ~35 min, and where thus reasonably close to the target values of pH 4.0 at about 13.5 min ($0.5 \times t_{1/2} + t_{feed}$) and pH 2.0 at about 27 min ($t_{1/2} + t_{feed}$). The pH of the remaining chyme at the end of the gastric digestion was 1.36 ± 0.28 and 1.24 ± 0.22 for the conditions with and without pepsin, respectively.

3.3. Dry matter content of the emptied digesta

Fig. 4A and 4B show the evolution of the dry matter content and the visual appearance of the digesta collected at the stomach exit, respectively. In Fig. 4A, the solid line represents the predicted evolution of dry matter content of emptied digesta under the assumption that the stomach behaves as a perfectly stirred reactor. The dashed line represents the same predictions but with the further assumption that casein particles cannot exit from the stomach. At 5 min, the dry matter content of the emptied digesta for the conditions with and without pepsin were not statistically different, and were close to the model prediction accounting

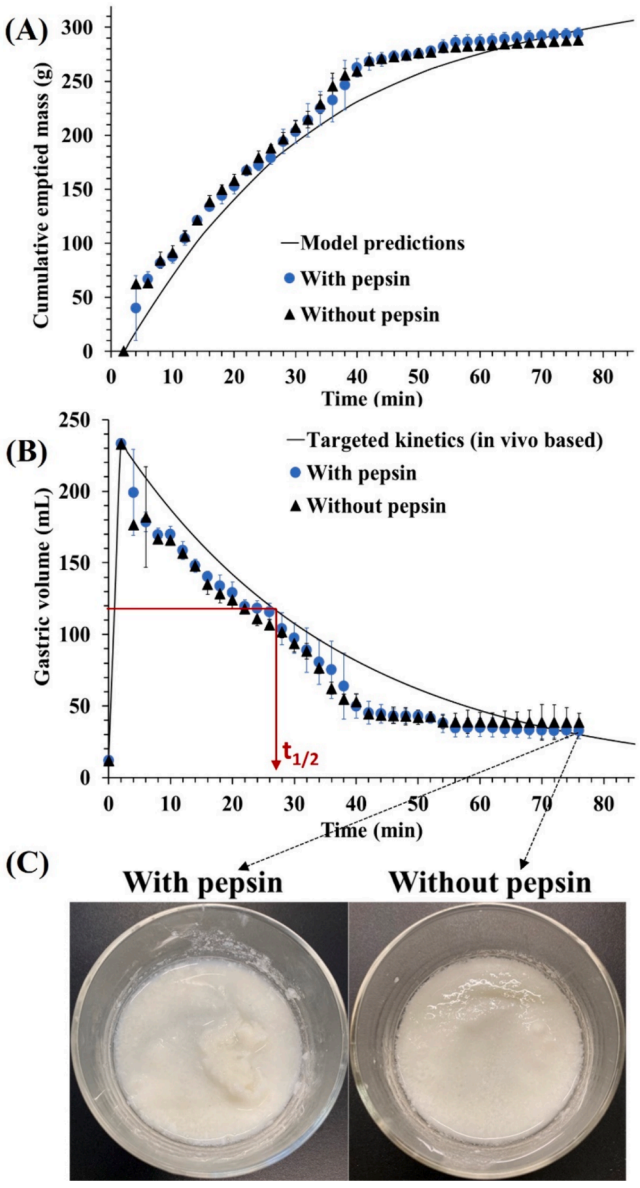

Fig. 2. Evolution of the cumulative mass of digesta emptied from the stomach **(A)**, of the gastric volume **(B)**, and visual appearance of the intragastric digesta at the end of the experiments **(C)** for the conditions with pepsin (●) and without pepsin (▲). Values are means ± SD over 3 replicates. In subplot **(A)**, the solid line represents the values predicted by modelling. In subplot **(B)**, the dotted line represents the targeted kinetics based on *in vivo* data.

for simple dilution effects (solid line). At t = 14 min, the decrease in the dry matter content was higher than expected from simple dilution considerations, and in a greater extent for the condition with pepsin than for the condition without pepsin, although not statistically different ($p > 0.1$). This statement was reinforced by the visual appearance of the collected samples (Fig. 4B) that appeared homogeneous and white in the absence of pepsin, only. From t = 20 to 52 min, all the results were well aligned with the dashed line, hence indicating that caseins were specifically retained within the stomach for both conditions in that period of time. This was confirmed by the visual aspect of the collected samples that all appeared translucent with a small amount of white precipitate that was attributed to casein particles. At t = 77 min, the dry matter content measured in the condition without pepsin continued to be fairly described by the model predictions assuming that caseins did not exit the stomach. However, in the presence of pepsin, the dry matter content increased substantially, leading to a significant higher value than that

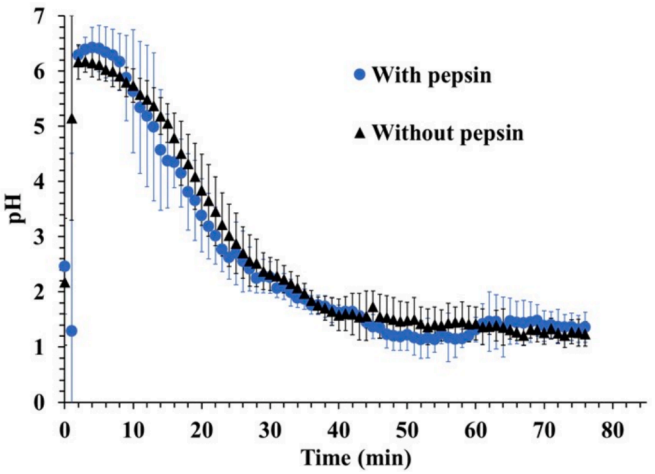

Fig. 3. Evolution of the intragastric pH for the conditions with pepsin (●) and without pepsin (▲). Values are means ± SD over 3 replicates.

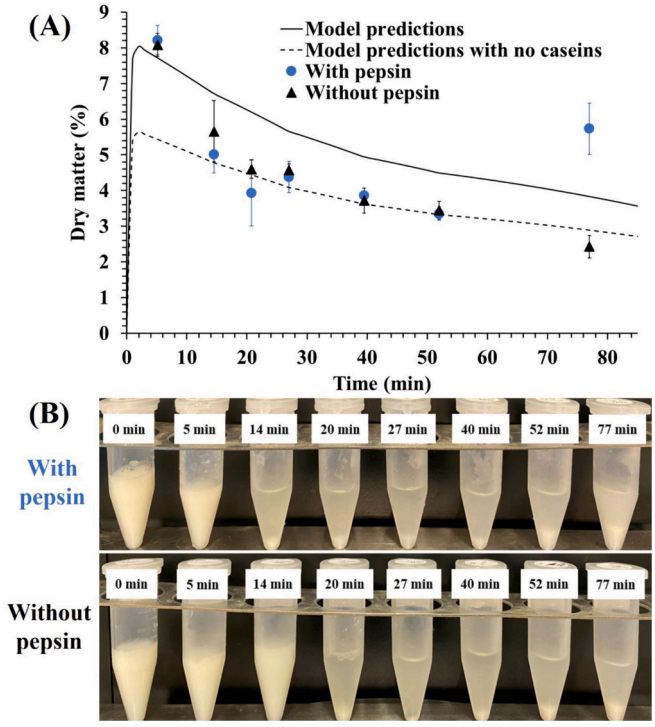

Fig. 4. Evolution of the dry matter content **(A)** and of the visual appearance **(B)** of emptied digesta for the conditions with (●) and without pepsin (▲). Values are means ± SD over 3 replicates. The solid line represents the values predicted by modelling under the assumption that the stomach behaves as a perfectly stirred reactor. The dashed line further assumes that caseins do not empty from the stomach.

without pepsin ($p = 0.04$), hence indicating that pepsin promoted the emptying of the casein particles retained in the stomach. This finding was consistent with the increased turbidity of the supernatant of the emptied digesta at t = 77 min in the presence of pepsin (Fig. 4B). The material remaining in the stomach at the end of the experiment had a significant lower dry matter percentage ($p < 0.02$) for the condition with pepsin (9.7 ± 0.5 %) than without (14.8 ± 2.0 %). This also led to a significantly lower dry mass of remaining material ($p < 0.002$) for the condition with pepsin (2.26 ± 0.26 g) than without (4.02 ± 0.32 g).

3.4. Macro- and microstructures of the digesta

Some of the optical microscopic images of the samples collected outside and inside the stomach are presented in Fig. 5. A more extensive view of the images acquired are provided in Fig. S1 of the Supplementary Material. At t = 5 min, all digesta were liquid without particles. At t = 14 min, milk particles were observed for both the conditions with and without pepsin, indicating that milk coagulation had started to take place in both conditions. In the absence of pepsin, light grey strings of particles were observed in the background, suggesting that the formation of milk particles was not yet complete. However, as these structures were not observed in the background with pepsin, it is likely that milk intragastric coagulation was complete in the presence of the gastric protease. From t = 14 min until the end of the experiment, it seemed that all the particles constitutive of the outside digesta had size of around 100 μm or below, whereas some larger particles were observed in the digesta collected inside the stomach (Fig. S1). This observation suggests that a small sieving effect took place during the gastric emptying of milk particles. At t = 77 min, many small particles could be observed with and without pepsin in the digesta collected both inside and outside the stomach. This demonstrates that the gastric biomechanics applied with the NERDT were strong enough to induce a mechanical breakdown of the dairy particles and promote their emptying. For the digesta collected at the stomach exit, it visually appeared that the particles were more fragmented in the condition with pepsin than without pepsin, an observation that would be consistent with the influence of protein hydrolysis by pepsin on gastric emptying.

3.5. Gastric emptying of proteins and protein hydrolysis

Fig. 6A and B show the protein profiles observed by SDS-PAGE of the samples collected at the stomach exit for the conditions with and without pepsin, respectively. The intensity of the casein bands was almost saturated at t = 5 min. It then decreased rapidly, from t = 14 min in the presence of pepsin, and from t = 20 min in the absence of pepsin. From t = 20 min to t = 52 min, almost no caseins were observed in the emptied digesta, hence demonstrating that caseins were specifically retained in the stomach during this period of time. Caseins were observed again at t = 77 min, with a higher band intensity and a wide range of peptides for the conditions with pepsin, as revealed by the appearance of a smear below the casein bands. The bands corresponding to α-LA and β-LG persisted throughout the experiments for the condition with and without pepsin, with a gradual decrease of their intensity.

Fig. 7A and 7B show the concentrations of caseins and whey proteins in the emptied digesta as estimated from 4th-derivative UV, noting that these values are independent of the extent of protein hydrolysis. The relative concentrations of intact caseins and β-LG extracted from an image analysis of SDS-PAGE gels (Fig. 6), are superimposed for comparison purposes. Both sets of results show that the concentration of caseins in the emptied digesta suddenly decreased at around t = 14 min and then gradually increased until the end of the experiment. The casein concentration at t = 14 min was higher in the absence of pepsin than in its presence, with $p = 0.07$ (trend) and $p = 0.004$ according to 4th-derivative UV and SDS-PAGE results, respectively. This suggests that casein coagulation occurred slightly earlier in the presence of pepsin. In the presence of pepsin, the casein concentration also increased significantly from t = 52 min and t = 77 min according to both kinds of measurements ($p < 0.05$), a phenomenon that was not observed in the absence of pepsin ($p > 0.8$). At t = 77 min, the casein concentration measured using 4th-derivative UV also tended to be higher in the presence of pepsin than in the absence of pepsin ($p = 0.07$). The decrease in the concentration of whey proteins (Fig. 6B) was more gradual and remained close to the model predictions accounting for simple dilution effects (solid line).

Fig. 7C presents the concentration of free NH_2 groups, which are carried by caseins, whey proteins and their peptides. This is the reason why the evolution of the free NH_2 concentrations showed an

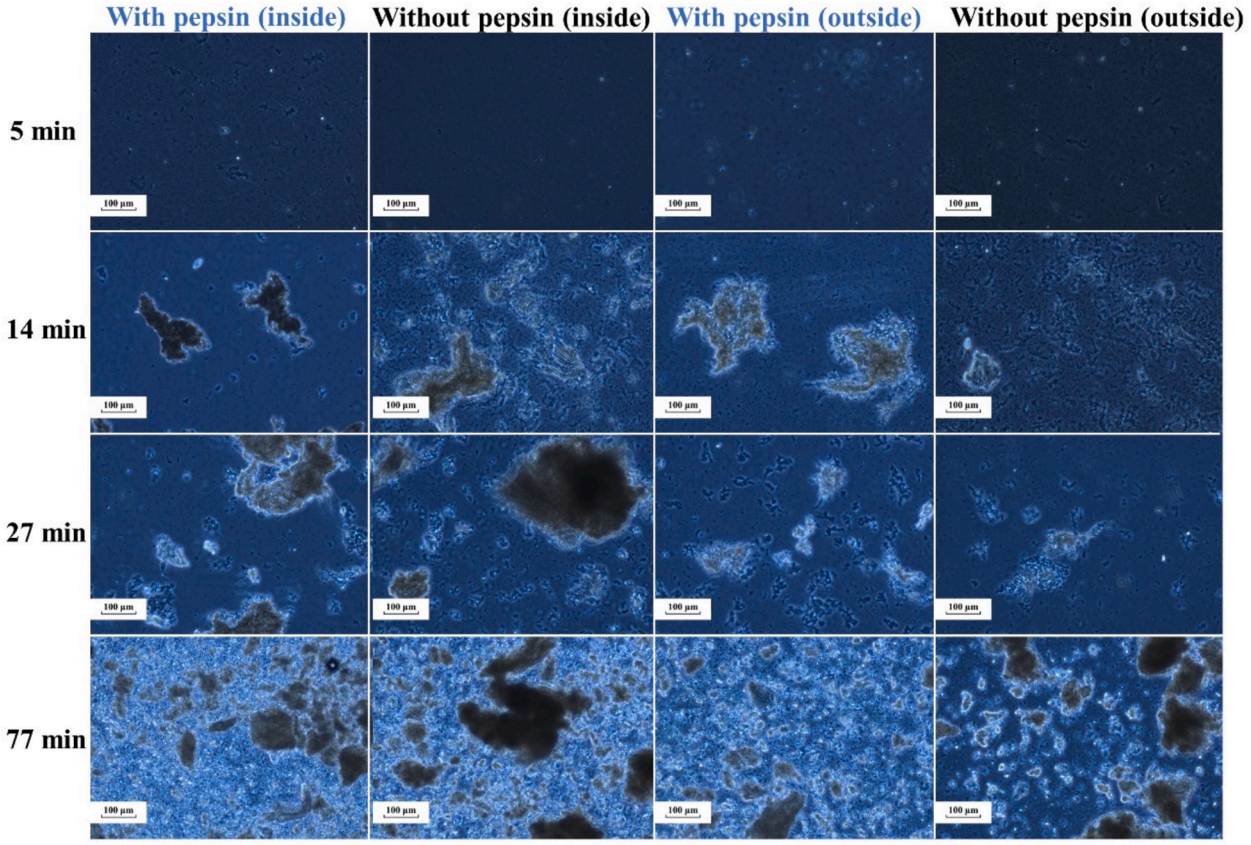

Fig. 5. Optical microscopy images (magnification x10) of the digesta collected inside the stomach and at the stomach exit for the conditions with and without pepsin. The images acquired at all time points are presented in the supplementary material (Fig. S1).

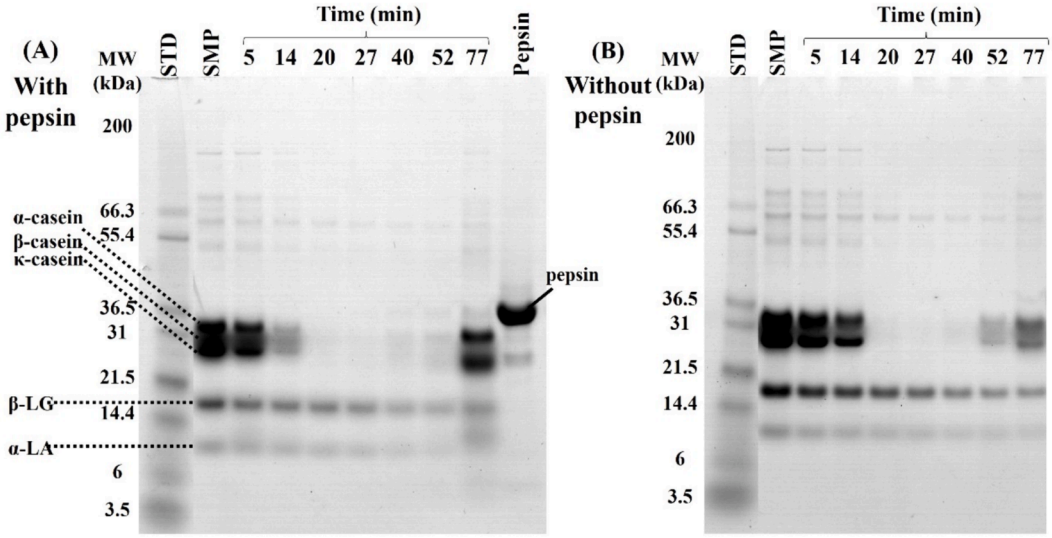

Fig. 6. SDS-PAGE profiles of the emptied digesta for the conditions with pepsin **(A)** and without pepsin **(B)**.

intermediate trend compared to that of whey protein and casein (Fig. 6A and 6B). The concentration of free NH_2 groups is also an indicator of the extent of protein hydrolysis by pepsin. However, from t = 5 min to t = 52 min, no significant differences were observed between the conditions with and without pepsin. From t = 52 min to t = 77 min, the concentration of free NH_2 groups tended to increase in the presence of pepsin ($p < 0.1$), leading to a high probability ($p = 0.06$) that more peptides were produced in the presence of pepsin than in its absence.

Overall, the concentrations of caseins, whey proteins and free NH_2 for the conditions with and without pepsin evolved similarly during the first 52 min and only started to diverge at the end of digestion, with some statistical trends and effects suggesting that a higher protein hydrolysis took place in the condition with pepsin.

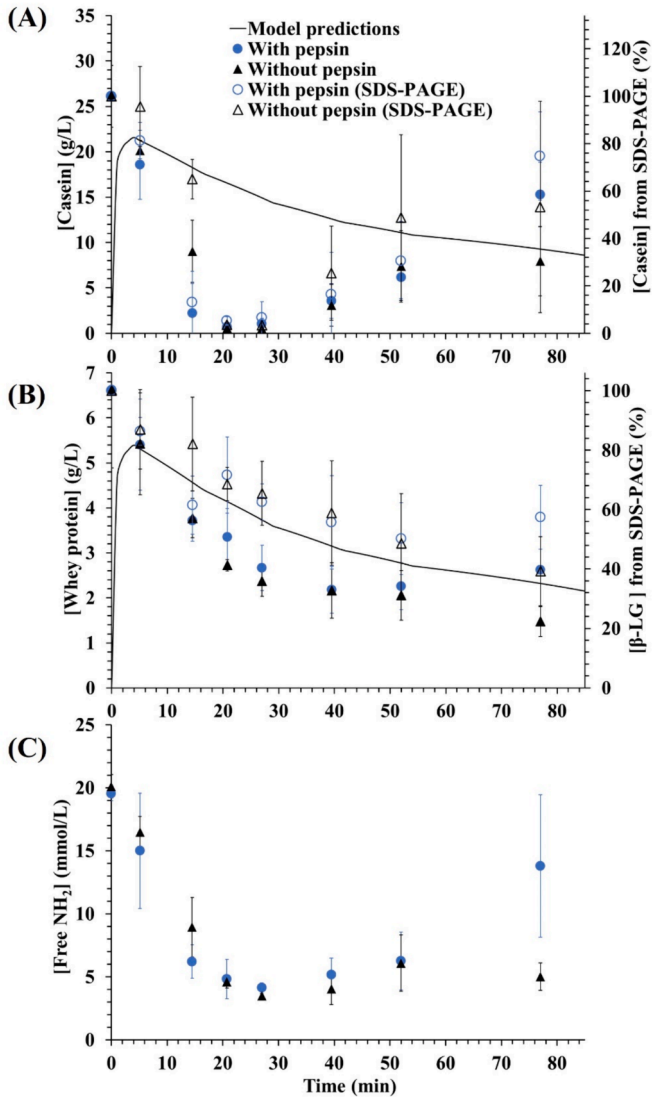

Fig. 7. Evolution of the concentrations of caseins **(A)**, of whey proteins **(B)** and of the free amines **(C)** in the emptied digesta for the conditions with pepsin (●, ○) and without pepsin (▲, △). Solid symbols represent the values measured using 4th-derivative UV, and open symbols represent the values estimated from SDS-PAGE results. Values are means ± SD over 3 replicates. The solid lines represent the values predicted by modelling.

4. Discussion

This study demonstrates the ability of the NERDT digestion system to investigate the gastric digestion of skimmed milk under physiologically relevant biochemical and biomechanical conditions. It allowed many aspects of the gastric behaviour of milk to be reproduced *in vivo*, in particular the rapid emptying of whey proteins and the delayed emptying of caseins.

Before discussing the mechanisms involved in the digestion of skimmed milk, it should first be emphasised that the NERDT operating parameters could be adjusted to reproduce well the target digestion conditions based on the *in vivo* literature. The evolution of the gastric volume closely followed the targeted exponential decay (Fig. 2B) based on the human study of Mahé et al. (1992), leading to an experimental $t_{1/2}$ (25.3 ± 2.1 min) very close the target value (27 min). Similarly, intragastric pH values of 4.0 and 2.0 were measured at t = 18 and 35 min, whereas these pH values should be reached at around 14 and 27 min (*i.e.* at 0.5 × $t_{1/2}$ and at $t_{1/2}$), respectively, as targeted from the results of Malagelada et al. (1979). Indeed, the gastric pH is not automatically regulated in the NERDT and the gastric emptying process relies on various parameters such as the opening size and frequency of the pyloric valve, or the tilting angle of the turntable on which the stomach model is mounted (Fig. 1). Although these considerations make the setting of the operating parameters more complex, such a gastric processing closely mimics the mechanisms taking place in the human stomach, reason why the NERDT offers a real potential to investigate the gastric behaviour of complex foods, such as milk, under fair biomimetic conditions.

The results obtained for the condition with pepsin are discussed below. During the first minutes of gastric digestion, the milk was liquid. At t = 5 min, the dry matter content (Fig. 4), the concentrations of whey proteins and caseins (Fig. 7) in the emptied digesta were therefore adequately predicted by our mass balance model assuming that the stomach behaved as a perfectly stirred reactor. Milk coagulated between t = 5 and 14 min under our experimental conditions with pepsin. From t = 14 min to about 40 min, the great majority of the casein content of the stomach could not be emptied (Fig. 7A), hence leading to a sudden decrease in the dry matter (Fig. 4) and free amine (Fig. 7C) content of the digesta collected at the stomach exit. It was also during this time period that the milk particles of the digesta inside the stomach appeared visually the largest, while the few particles observed at the stomach exit appeared comparatively smaller (Fig. 5 and Fig. 1S). From t = 40 min, the concentrations of casein products in the emptied digesta gradually increased (Fig. 7A). According to our 4th-derivative UV measurements, it reached 15.3 ± 3.6 g/L at t = 77 min, a value that can be compared with the 0.7 ± 0.5 g/L measured at t = 20 min. This increase in the concentration of emptied casein products at the end of the experiment, which included many peptides (Fig. 6A), is in very good accordance with the sudden increase in the number of small and fragmented particles observed in the emptied digesta (Fig. 5 and Fig. S1). These observations witness the breaking down of milk particles by the combined action of pepsin and the gastric biomechanical contractions.

This chronology of events is consistent with the scientific literature. As recently reviewed by Huppertz & Chia (2021), both *in vivo* and *in vitro* studies have shown that unheated or mildly heated milk coagulates rapidly after ingestion under the combined action of pepsin and pH, typically in less than 30 min. For instance, an MRI study on women fed with whole bovine milk reported that coagulation started within 10–20 min, with a strong coagulum forming after 30 min (van Eijnatten et al., 2024). It is also known that in mildly heated milk, as used in the present study, whey proteins are not directly incorporated in the casein curd (Ye et al., 2016), which is why they continue to be emptied from the stomach in a regular flow, whereas caseins are specifically retained. This phenomenon is even at the origin of the concept that whey proteins and caseins can be considered as 'fast' and 'slow' digested proteins, respectively (Boirie et al., 1997). In the late stages of digestion, the combined action of pepsin and of biomechanical contractions allowed more casein proteins and peptides to be emptied from the stomach, leading to an overall slow rate of casein nitrogen delivery to the small intestine (Fig. 6A) as observed in humans (Mahé et al., 1996).

The second aim of this study was to evaluate the influence of pepsin on the gastric coagulation and emptying of milk proteins. The skimmed milk coagulated faster in the presence of pepsin than without according to the higher concentrations of caseins measured at the stomach exit at t = 14 min by 4th-derivatuve UV ($p = 0.07$) and SDS-PAGE ($p = 0.004$). This was also supported by the visual appearance of the collected samples, which, at this time, were still white (Fig. 4B) and not yet fully coagulated (Fig. 5) in the absence of pepsin. This finding further confirms the known influence of pepsin in the gastric coagulation of milk (Ye et al., 2016; Mulet-Cabero et al., 2019). We may, however, acknowledge that the absence of pepsin did not delay much the gastric coagulation of milk in comparison to previously reported data. This is because the acid coagulation of milk takes place around pH 5 (Lucey & Fox, 1992), a value that was reached in about 15 min in our experimental conditions in the absence of pepsin (Fig. 3). In the study of Ye

et al. (2016), in which gastric acidification and emptying were much slower ($t_{1/2}$ of about 45 min), the gastric coagulation of milk in the presence of pepsin was visible after 8 min at pH 6, whereas it only appeared after 40 min and pH 5 without pepsin. In the study of Mulet-Cabero et al. (2019), in which the gastric acidification and emptying was even more slow ($t_{1/2}$ of about 100 min), the gastric coagulation of milk in the presence of pepsin was reported to be visible within the first 10 min at pH 5.5–6, and only after 35 min at pH 5 in the absence of pepsin. These examples illustrate the difficulty to assess the true contribution of pepsin in the gastric coagulation of milk; a mechanism that can hardly be investigated *in vivo* and that is mostly determined by the experimental conditions used *in vitro* (*e.g.* kinetics of gastric pH, of gastric emptying and of pepsin addition).

With regard to the extent of proteolysis performed by pepsin after milk coagulation, the evolution of the concentrations of dry matter, caseins, whey proteins and free NH_2 groups was not affected by the presence or absence of pepsin from t = 20 to 52 min in our experimental conditions. The effects of pepsin action were only observed towards the end of the digestion experiments, at t = 77 min, on a number of variables that includes the casein concentration measured by both methods and the free NH_2 concentration, as well as on the dry mass of the material remaining in the stomach. This contrasts somehow with the results of Mulet-Cabero et al. (2019) who observed a significant increase of free NH_2 groups per gram of proteins at their first sampling time, at t = 35 min, which plateaued after ~ 100 min, when the $t_{1/2}$ they considered was reached. This discrepancy can also be explained by the different experimental conditions used in both studies. Indeed, when we targeted to reach a pepsin activity of 2000 U/mL after two $t_{1/2}$, the same level of pepsin activity was undoubtedly reached much earlier, presumably before $t_{1/2}$, in the study of Mulet-Cabero et al. (2019). According to the study of Nau et al. (2022), who mapped the pepsin concentration and the extent of proteolysis in the stomach of pigs fed egg-white gels over 6 h postprandial period, a long delay may actually be needed before a significant level of intra-gastric proteolysis is reached. Although the pigs were fed with an important quantity of meal in that study, the authors did not observe any significant increase in proteolysis before 6 h of digestion, which corresponds to about two $t_{1/2}$ in their conditions. These considerations actually call for more *in vivo* data on the rate of pepsin secretion.

Finally, the usefulness of combining a modelling approach together with the analysis of samples that are collected at the stomach exit, rather than collected from inside, can be highlighted. Indeed, samples collected from the inside of the stomach with a cut pipette were also considered and analysed during the experiment. However, it rapidly appeared that the measurements on those samples were associated with a huge variability linked to the difficulty to collect a representative sample from the heterogeneous contents of the stomach. This is the reason why these data were not shown in the present paper except for the microscopic observations. The samples collected at the stomach exit exhibited less variability because of the sieving effect of the stomach model of the NERDT. Obviously, these samples do not directly reflect the intragastric contents and the interpretation of the results is less straightforward. In this context, the modelling approach developed proved to be very valuable to deepen our interpretation of the experimental data. Such a combined *in vitro-in silico* approach could be used in future studies to further optimize and validate that the sieving effect produced by the NERDT can adequality mimic the physiological reality before investigating the behaviour of heterogeneous gastric contents, as typically encountered after the ingestion of complex foods or real meals.

5. Conclusion

This study has evaluated the suitability of the NERDT as a digestion system to replicate the *in vivo* gastric digestion behaviour on skimmed milk. A set of operating parameters allowing to reproduce target kinetics of gastric emptying and acidification were obtained after some preliminary experiments and with the help of a mass balanced model. In our experimental conditions, the intra-gastric coagulation of milk took place after about 10 min of digestion, and a sieving effect leading to a delayed emptying of casein particles was subsequently observed. The combined action of pepsin and of the biomechanical contractions allowed more casein proteins and peptides to be emptied from the stomach after more than one hour of gastric digestion. These results compare well with the *in vivo* literature but also suggest that the dynamic evolution of pepsin activity, for which *in vivo* data are scare, is key to properly understand and reproduce the slow gastric emptying of casein particles and their peptides. Overall, the NERDT shows a great potential to deepen our understanding on the gastric behaviour of complex foods and meals with consideration of realistic particle size and gastric content heterogeneity.

CRediT authorship contribution statement

Jiajun Feng: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Ines Greco:** Writing – review & editing, Methodology, Conceptualization. **Olivia Ménard:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Jeehyun Lee:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Romain Jeantet:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Didier Dupont:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Steven Le Feunteun:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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