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Feasibility of using a realistic food bolus for semi-dynamic *in vitro* gastric digestion of hard cheese with pH-stat monitoring of protein hydrolysis

Martine Morzel *, Sylvaine Ramsamy, Steven Le Feunteun

INRAE, Institut Agro, STLO, 35042 Rennes, France

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ABSTRACT

Oral processing of solid foods leads to boluses made of a human saliva and particles distributed in the size range ~ 0 to 5 mm. However, studies on the release of nutrients from realistic solid food boluses during digestion are scarce because such mechanisms are difficult to investigate *in vivo*, and *in vitro* experiments generally recommend to extensively mince solid foods during the oral stage. Similarly, it has previously been shown that the peptic hydrolysis of protein solutions during *in vitro* gastric digestion can be monitored by acid titration in both static and dynamic pH conditions, but such approach has never been evaluated in the presence of particles of several millimetres in size. The first objective of the study was therefore to test the feasibility of using a realistic food bolus for gastric digestion studies with a pH-stat monitoring of proteolysis, using Emmental cheese as a solid food and with consideration of gastric acidifying kinetics. Degree of hydrolysis (DH) of proteins was monitored from two series of experiments performed in the presence and absence of pepsin. Other DH measurements, estimated from an independent approach based on the amount of free NH_2 groups (OPA method) contained by peptides released in the supernatant (UV absorbance) validated the pH-stat results. A second objective of this work was to test the possible influence of human saliva on gastric proteolysis (in comparison with a water-based bolus). Results showed that saliva slightly delayed initiation of proteolysis, which could be explained by the slightly higher initial pH of the saliva-based bolus, but had no statistical effects on pepsin activity. We conclude that acid titration with a pH-stat system can be a valuable approach to monitor the gastric *in vitro* proteolysis of realistic solid food boluses in dynamic pH conditions.

1. Introduction

pH-stat titration is a simple and rapid method to follow enzyme activity during *in vitro* food digestion experiments. pH-stat titration is most commonly employed to follow lipolysis during the intestinal phase of digestion (Li et al., 2011) but it was also shown to be suitable to monitor pepsin activity during gastric digestion (Mat et al., 2018). This method was successfully tested on a protein solution in both static and dynamic pH conditions (Mat et al., 2018), as well as on liquid or gelled emulsions comminuted into sub-micromillimeter particles in static *in vitro* conditions (Mat et al., 2020). This preparation of the gel samples is consistent with the recommendations of the INFOGEST *in vitro* static and semi-dynamic protocols (Brodkorb et al., 2019; Mulet-Cabero et al., 2020) where the oral phase of digestion is performed by homogenizing the food with a simulated salivary fluid. In case of solid foods, mixing or mincing should be applied, and water added if necessary in order to obtain « a consistency similar to tomato paste or mustard ». This consistency is quite far from what is observed in food boli collected at the point of swallowing in humans for a variety of solid foods. Indeed, real food boli are made of particles of various sizes impregnated with saliva, which results in a cohesive but heterogeneous material. For example, particle size was monitored in a variety of food products (Jalabert-Malbos et al., 2007) using the sieving method. For Emmental cheese, approximately 25% and 20% of the bolus mass were constituted of particles below 1 mm and above 4 mm, respectively. The extensive grinding of solid foods prior to gastro-intestinal *in vitro* digestion can mask some effects of physiological relevance such as acidity and moisture uptake by foods in the stomach (Mennah-Govela et al., 2020) or delayed enzyme actions onto microstructurally intact food fragments (Grundy et al., 2016). However, the presence of large particles is usually considered as a challenge in digestion studies since they may impair the collection of a representative sample and/or subsequent analyses. As the pH-stat method does not rely on sample collection, it may represent a suitable means to investigate the kinetics of gastric proteolysis of

* Corresponding author.
E-mail address: martine.morzel@inrae.fr (M. Morzel).

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realistic food boluses. However, there are no reports of this method being applied in presence of particles of several millimetres in size. The general objective of this work is therefore to test whether the pH-stat method coupled to semi-dynamic *in vitro* digestion is compatible with a food bolus showing realistic features in terms of particles size distribution. To better simulate the oral phase further, commercial human saliva was also used and, as a secondary objective, we evaluated whether proteolysis during gastric digestion differed between saliva-based and water-based boli.

2. Materials and methods

2.1. Preparation of food boli

Emmental cheese containing 28% lipids, 28% proteins and 1% carbohydrate (w/w) was ground in a blender (8010S, Waring Laboratory Science) twice for 10 s. The resulting particles were sorted by size using sieves of aperture 4, 2 and 1 mm. Large cheese cubes of approximately 5 mm of edge were also produced with a knife. Cheese particles from the 4 fractions (<1 mm, 1–2 mm, 2–4 mm, and 5 mm cubes) were then combined in mass proportions of 10:40:30:20 to reproduce the bolus particle size distribution reported for Emmental in humans (Jalabert-Malbos et al., 2007). Aliquots of 8 g of such mixed-size particles were stored at −25 °C. On the day of the digestion experiment, aliquots were defrosted and then mixed manually with 8 mL of either pooled human saliva (Lee Biosolutions, United States) or distilled water. This 1:1 cheese to saliva ratio corresponds to the upper end of the range of saliva incorporation in cheese recorded on healthy adults (Repoux et al., 2012).

2.2. Semi-dynamic gastric digestion with pH-stat monitoring

Porcine pepsin (P6887) and the protease inhibitor Pepstatin A (P5318) were obtained from Sigma-Aldrich (France). Pepsin activity was determined according to the protocol described in (Minekus et al., 2014). Digestion experiments were performed in triplicate using a procedure based on the semi-dynamic *in vitro* digestion protocol of INFOGEST consortium with two adaptations. First, the food bolus was constituted as described above. Second, gradual gastric emptying was not considered.

Practically, a double-wall 20–90 mL glass vessel with truncated cone (Metrohm) maintained at 37 °C by circulating water was used. The vessel was equipped with a lid with openings to monitor the pH using a titration unit (842 Titrando, Metrohm). About 20 min before the start of the digestion experiments, 8 mL of water (as a drink) and 1.9 mL of simulated gastric fluid (SGF) at pH = 2 were added to the vessel and let to warm. Just before the addition of the bolus, 0.2 mL of a freshly prepared pepsin solution (42,000 U/mL in SGF) kept on ice was also added. The bolus was placed into the glass vessel and magnetic stirring was started. The pH electrode of the titration unit was then immersed and the pH recording was started with a data acquisition frequency of 1 s^{-1}. Gastric secretion was simulated by the delivery of SGF at 400 mM HCl at a constant rate of 0.28 mL/min, and 6 additions of 300 μL of pepsin at 42,000 U/mL in SGF at 15 min-intervals. This procedure allowed to reach pH ~ 2 and pepsin activity of 2,000 U/mL in a volume of 42 mL at about t = 92 min. The gastric phase continued until t = 120 min.

For the purpose of calculating degrees of hydrolysis, similar experiments were conducted in absence of pepsin. In total 10 digestion experiments were performed: 4 in absence of pepsin (2 in absence of saliva, 2 in presence of saliva,) and 6 in presence of pepsin (3 in absence of saliva, 3 in presence of saliva). Samples are noted P-S-, P-S+, P + S- and P + S+, respectively.

2.3. Sampling

Immediately after the first pepsin addition (t = 0), or at the equivalent time in absence of pepsin, and every 15 min up to 120 min, samples were taken for subsequent biochemical analyses in the following manner: magnetic stirring was interrupted for approximately 15 s to let the larger particles settle, and 1 mL of the upper aqueous phase was sampled. For the assays containing pepsin, 10 μL of the proteolysis inhibitor pepstatin A (0.5 g/L diluted in methanol/acetic acid 9:1) were added to the samples.

2.4. Measurement of the degrees of hydrolysis (DH) of proteins

2.4.1. DH from the pH titration method

Data obtained by pH stat were converted into DH of cheese proteins over the entire digesta using the equation (Mat et al., 2018):

$$DH(\%) = 100 \times \frac{C \times (V_{P+}(pH) - V_{P-}(pH))}{m_{prot}^{food} \times h_{pb}^{prot}} \times \frac{1}{1 - \alpha_{COOH}(pH)} \quad (1)$$

where C is the concentration of the HCl titrant (0.04 mmol/mL), $V_{P+}(pH)$ the volume (mL) of titrant added in presence of pepsin at a given pH, $V_{P-}(pH)$ the volume (mL) of titrant in absence of pepsin at the same pH (mean of the 2 replicates), m_{prot}^{food} the mass (g) of cheese proteins, h_{pb}^{prot} the quantity of peptide bonds per mass of protein (mmol/g), and $\alpha_{COOH}(pH)$ the mean degree of dissociation of the carboxylic groups produced at the considered pH. h_{pb}^{prot} was set at 8 mmol/g (Adler-Nissen et al., 1983), and α_{COOH} was set at 0 in accordance with the study of (Mat et al., 2018) who showed that this approximation enables fair estimates of DH during the course of acidification with a negligible error for pH ≤ 3.

All calculations were made using Matlab® software (The MathWorks Inc., Natick, USA). The main steps of the data treatment procedure were as follows. Recorded pH values were first smoothed with a moving average on 60 points, corresponding to a 1 min interval. Means and standard deviations of the titrant volumes and of the smoothed pH values were then calculated as a function of time over the replicates for each set of experiments. The times and volumes were, thereafter, linearly interpolated to the mean pH values recorded during the P + S- experiments to enable converting titrant volumes into degree of hydrolysis with Eq. (1). Indeed, Eq. (1) can only be solved with identical pH values for both P + and P- experiments, not times.

2.4.2. DH from the OPA method and the absorbance at 280 nm

Samples taken during digestion were immediately centrifuged at 5,000g for 10 min at 4 °C. The supernatant was filtered through a membrane with 0.8 μm pores to remove lipids and its pH was raised to approximately 7 with NaOH. The solution was then analysed for both its concentration in free NH_2 groups using the o-phthaldehyde (OPA) method and its concentration in peptides using the UV absorbance at 280 nm.

Free NH_2 groups were measured by the OPA method adapted from (Church et al., 1985). 100 mL of reagent was prepared with 2.5 mL of OPA (10 mg/mL in ethanol), 2.5 mL of 20% SDS, 50 μL of β-mercaptoethanol and 95 mL of 20 mM sodium tetraborate. 96-well UV clear plates and a MultiskanTM GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, USA) were used to measure the absorbance at 340 nm after 10 min incubation of the diluted samples (50 μL) in the OPA reagent (100 μL). A calibration curve was established using L-methionine as a standard (0–2 mM). Results were expressed in μmol/L of NH_2 in the supernatant, and the total amount of NH_2 groups in the supernatant was calculated. Each sample was measured in triplicate.

The peptide concentration was measured from the absorbance of aromatic amino-acid residues at 280 nm in a 10 mM K_2HPO_4 buffer using quartz cuvettes and a UV spectrometer (UVmc^2, Safas monaco, Monaco, France). A calibration curve was established using a hydrolysate of our Emmental cheese as a standard (0–1.5 g/L) that was produced with alcalase (until full disappearance of cheese particles after an

overnight reaction). Results were expressed in g/L of peptide in the supernatant, and the total mass of peptide in the supernatant was calculated.

The degree of hydrolysis of the peptides released in the supernatant (DH_{sup}) can be estimated according to:

$$DH_{sup}(\%) = 100 \times \left(\frac{n_{NH_2}^{pepP+} - n_{NH_2}^{protP+}}{m_{pep}^{P+} \times h_{pb}^{prot}} \right) \quad (2)$$

where $n_{NH_2}^{pepP+}$ is the quantity (mmol) of NH_2 groups contained in the peptides released in presence of pepsin, $n_{NH_2}^{protP+}$ the quantity (mmol) of NH_2 groups that would be contained in the equivalent mass of unhydrolyzed proteins, m_{pep}^{P+} the mass (g) of released peptides in presence of pepsin, and where h_{pb}^{prot} has the same meaning and value as in Eq. (1). Eq. (2) was solved by further considering that:

$$n_{NH_2}^{protP+} = m_{pep}^{P+} \times h_{NH_2}^{prot} \quad (3)$$

with $h_{NH_2}^{prot}$ the quantity of NH_2 groups per mass of unhydrolyzed proteins, taken as 0.523 mmol/g for caseins (Bo et al., 2019),

and

$$n_{NH_2}^{pep\ P+} = \Delta n_{NH_2}^{P\pm} + m_{pep}^{P-} \times h_{NH_2}^{prot} \quad (4)$$

with $\Delta n_{NH_2}^{P\pm}$ the difference between the released quantities (mmol) of NH_2 groups in presence and absence (mean of the 2 replicates) of pepsin, and m_{pep}^{P-} the mass (g) of released peptides in the absence of pepsin (mean of the 2 replicates). The second term of Eq. (4) allows the consideration of the NH_2 groups contained in the peptides and/or proteins that are released form the cheese matrix in the absence of pepsin.

The degree of hydrolysis of proteins over the entire digesta (*DH*) was further estimated under the assumption that the peptides produced upon pepsin action were all released in the supernatant, according to:

$$DH(\%) = \left(\frac{m_{pep}^{P+}}{m_{prot}^{food}} \right) \times DH_{sup} \quad (5)$$

As later explained, it appeared that the DH of the peptides released in the supernatant (DH_{sup}) remained relatively stable from the start of pepsin action (observable from 30 min in our data) until the end of the digestion experiments, though with a relatively high variability over the 21 estimates within each condition (3 replicates × 7 time points). Eq. (5) was thus solved by considering the mean DH_{sup} values obtained for the P + S- and the P + S + conditions (i.e. over 21 estimates for each condition).

2.5. Statistics

The difference between the conditions without or with saliva was tested by the non-parametric Wilcoxon test for the values « NH_2 groups in the supernatant », « peptide masses in the supernatant », and « DH calculated from spectrophotometric data ».

3. Results

3.1. pH evolution during pH-stat digestion

The kinetics of pH drop during the pH-stat digestions are presented in Fig. 1. In the presence of saliva, the initial pH was higher (~6.1) than when only cheese was present (~5.6). In the saliva-containing experiments, pH 5.6 was reached after 2 min. The pH drop in saliva-containing experiments was therefore slightly delayed throughout digestion, but the curves followed the same trend as their equivalent with no saliva.

The pH drop was almost identical for the four conditions (P ± S ±) between 2 and approximately 25–30 min, i.e. when the pH reached 4.4–4.3, after which the pH in pepsin-containing experiments was systemically higher than in experiments with no pepsin added. This resulted in a lower total volume of HCl added in absence of pepsin. Thus, at the end of the 120-min digestion, the mean volumes of titrant added were 18.5 and 19.1 mL in P-S- and P-S + conditions *vs* 20.6 and 21 mL in P + S- and P + S + conditions, respectively.

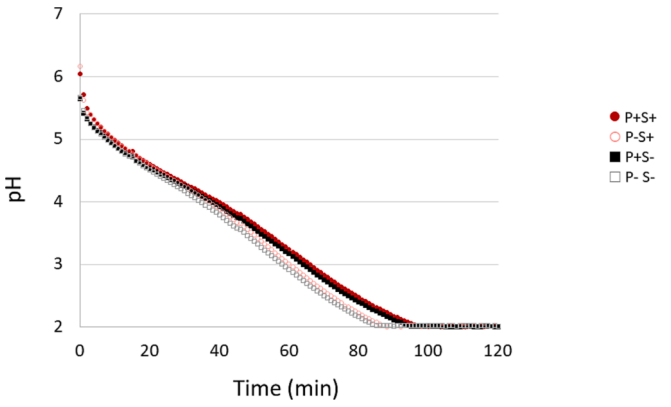

Fig. 1. pH evolution during pH-stat digestions of cheese in presence or not of pepsin and human saliva. n = 3 in presence of pepsin, n = 2 in absence of pepsin.

3.2. Degree of hydrolysis of cheese proteins measured by pH-stat

Fig. 2 illustrates the DH calculated from pH titration data. There were slight differences between the two conditions, namely that DH started to increase after approximately 6 min in absence of saliva *vs* 12 min in presence of saliva. Because of this delay in the initial rise in DH values, DH was slightly lower (approximately −0.1 to −0.3 point) in presence of saliva at a given time. Nevertheless, the slope of DH increase was similar in both conditions. At the end of gastric digestion, DH (%) was 4.51 ± 0.37 and 4.27 ± 0.15 in the S- and S + experiments, respectively.

3.3. Peptides and NH_2 groups in digesta supernatant

The mass of peptides (mg) and the quantity of NH_2 groups (μmol) measured in the digesta supernatant based on the absorbance at 280 nm and on the OPA method, respectively, are presented in Fig. 3. The mass of peptides rose for all conditions from 0 to 15 min. Because proteolysis is not expected during that time, where pH conditions are unfavourable to pepsin activity, this increase is most probably due to a solubilization of peptides (and possibly other compounds) present in the cheese under the effect of stirring. This is confirmed by the fact that no substantial

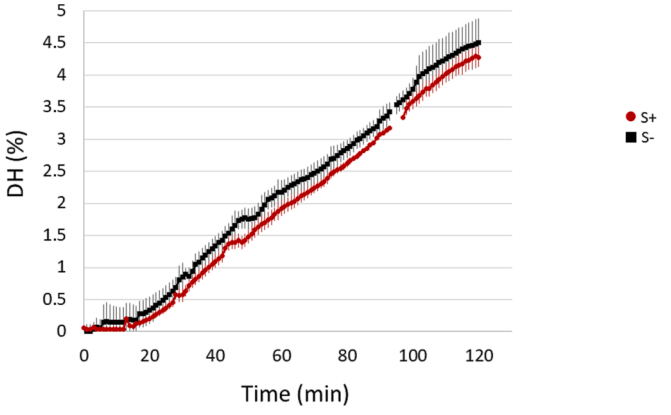

Fig. 2. Degree of hydrolysis of cheese proteins calculated from pH titration data (pH-stat method). n = 3 per condition.

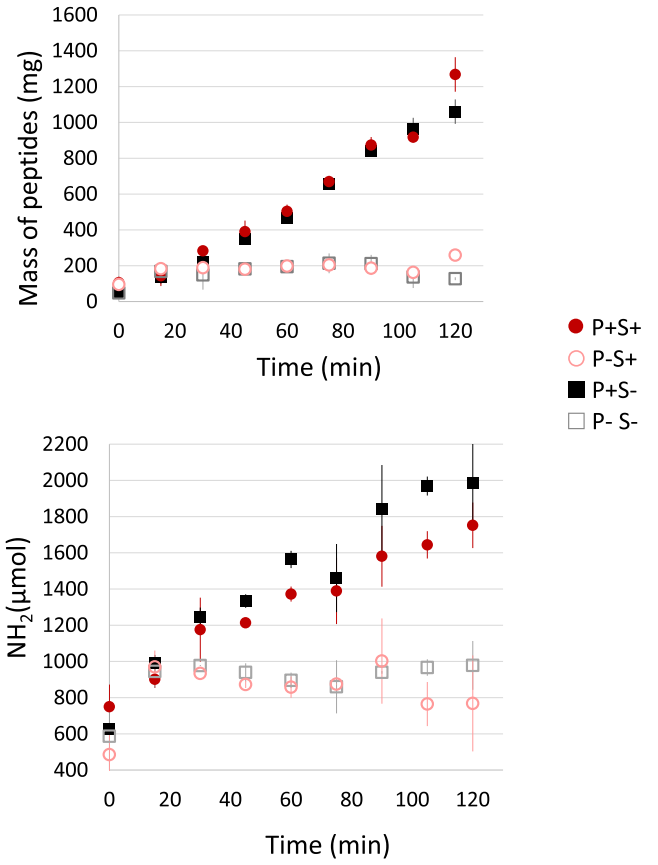

Fig. 3. Peptide mass in mg (top) and quantity of NH_2 groups in µmol (bottom) in the digestion supernatants of 8 g of cheese in presence or not of pepsin and human saliva. n = 3 in presence of pepsin, n = 2 in absence of pepsin.

change could be observed during the first 15 min in both the released mass of peptides and quantity of NH_2 groups when no pepsin was added. In the pepsin-containing conditions the mass of peptides and quantity of NH_2 groups increased over digestion and diverged from the pepsin free conditions from 30 min on. There was no significant difference between the two conditions S- and S + for the peptide mass at any time point, while the abundance of NH_2 groups was generally lower (but overall not significantly) in saliva-containing samples.

The increase in NH_2 groups between the P + and P- conditions normalized by the corresponding increase in the peptide mass is indicative of the DH of the peptides released in the supernatant. This ratio remained approximately constant over time from 30 to 120 min in both conditions, with average values of 1.16 ± 0.26 µmol/g and 0.86 ± 0.26 µmol/g in absence and presence of saliva, respectively. According to Eq. (2), these values correspond to mean DH (%) in the supernatant of 10.33 ± 4.07 and 6.60 ± 3.90 in absence and presence of saliva, respectively.

3.4. Degree of hydrolysis calculated from spectrometric data

Fig. 4 shows the DH over the entire digesta as calculated from spectrometric data and Eq. (5). DH increased regularly over time, with average values systematically lower in presence than in absence of saliva (but overall not significantly). The final DH (%) was 4.08 ± 2.06 and 3.12 ± 0.93 for the S- and S + conditions, respectively.

4. Discussion

The first objective of the study was to test the feasibility of using a realistic food bolus, thus containing some large particles, for gastric digestion studies with a pH-stat monitoring of proteolysis. Overall, no

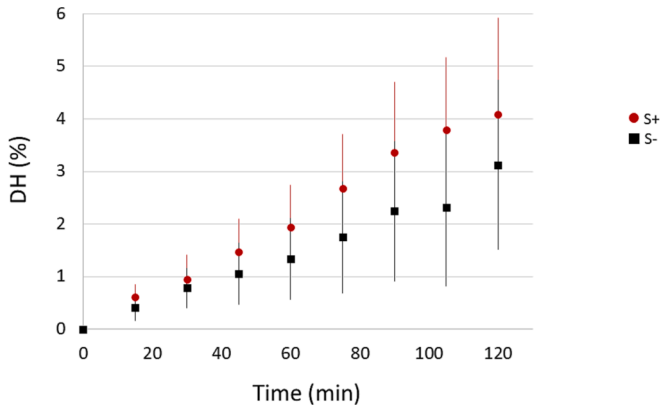

Fig. 4. Degree of hydrolysis of cheese proteins calculated from spectroscopic results (OPA measurements and Absorbance at 280 nm). n = 3 per condition.

major technical difficulties were encountered to evaluate the degrees of protein hydrolysis by pH-titration. Although more prone to experimental errors and not exempt of assumptions, the DH estimated from biochemical analyses of digesta were in the same range of values, hence providing a fair independent validation of the DH measured with pH-stat.

Using realistic cheese bolus particle sizes (median size of 2–3 mm), (Fang et al., 2016) and (Tran Do and Kong, 2018) have also investigated the *in vitro* gastric digestion behaviour of some Cheddar, Parmesan, and Mozzarella using static and dynamic *in vitro* protocols, respectively. Although they did not evaluate the DH of proteins during gastric digestion, these studies reported that the amount of peptides (or of solid material) released in the supernatant by the end of the gastric digestion were in the range of 40–60% for 6 cheeses over the 7 they studied. In spite of important differences in the digestion protocols, this order of magnitude is in very good agreement with the 1.06 ± 0.07 g (P + S-) and 1.27 ± 0.10 g (P + S +) of released peptides we obtained (Fig. 3), which correspond to 47% and 57% of the total amount of Emmental proteins to be digested (2.24 g), respectively.

Unfortunately, it is more difficult to compare our DH results with the literature on the gastric digestion of cheese because such values are scarce. The final DH found in the present study (from 3.4 to 4.5% depending on methods and conditions) are, however, rather consistent with the DH measured in other types of solid gels. For example, after 3 h of peptic digestion in a device conceived to simulate the peristaltic movement of the stomach, final DH of 2.5% and 7.9% were obtained for an egg white gel and a 15% whey protein gel, respectively (Luo et al., 2015). In another study, after 2 h of gastric digestion in a semi-dynamic model, DH were 7.3% and 3.1% for whey protein gels at 15% or 20%, respectively (Deng et al., 2022). Final DH ranging from 5 to 8% were also recorded at the end of static *in vitro* gastric digestion for 12% soy protein gels (Zhao et al., 2020) and 15% whey protein gels (Mat et al., 2020). The final values we obtained are also consistent with previous reports on the peptic hydrolysis of caseins, for which DH of approximately 5%, 8% and 10% were obtained for solutions of α-caseins (Monogioudi et al., 2011), a suspension of micellar casein microaggregates (Salelles et al., 2021) and a solution of κ-caseins (Sheng et al., 2022), respectively. The protein fraction of cheese is made mainly of caseins, but lower values than those reported in solutions are expected. This is for example consistent with findings of (Luo et al., 2015), who attributed the differences between the digestion of protein gels and solutions to immobilisation of the substrate in the gel network and steric hindrance for pepsin ingress inside the particles and for peptides release.

The second objective of this work was to evaluate whether the use of human saliva in the food bolus had an influence on proteolysis compared to the use of water. We found that saliva delayed to a small extent the initiation of proteolysis, which can easily be explained by the

higher initial pH of the saliva-based bolus. Thus, saliva collected at the exit of major salivary glands is mildly acidic (Neyraud et al., 2009) but its pH rises to neutrality or slightly above as soon as it becomes in contact with air (Bardow et al., 2000). Mixing saliva with cheese, whose pH was around 5.6, thereby increased the pH of the bolus compared to a water-based bolus. In the conditions of gradual pH decrease used in this study, it took slightly longer to reach pH conditions favourable to pepsin activity.

Besides this small timing difference, the concentrations of NH_2 groups in the supernatant appeared slightly reduced in presence of saliva. However, the difference was not significant considering the entire digestion duration. Human whole saliva is a very complex fluid containing more than 5,000 different proteins and peptides of human and bacterial origin (Grassl et al., 2016), including proteolytic enzymes such as lactotransferrin, kallikrein-1 or human airway trypsin-like protease (Sun et al., 2009). Autoproteolysis of some proteins during storage is very well-described in saliva, and resilience to protease inhibition by a mixture of six inhibitors illustrates that whole saliva contains a unique mixture of enzymes (Thomadaki et al., 2011). Nevertheless, the quantity of proteases brought by saliva can only be minute and can be considered as negligible in comparison to the quantity of pepsin used in *in vitro* digestion. Saliva also contains different types of protease inhibitors, the most abundant being cystatins SA and SN which are potent inhibitors of cysteine proteases (Baron et al., 2008). This activity is clearly irrelevant in the context of gastric digestion, pepsin being a serine protease. Therefore, the small reducing effect of saliva on proteolysis, if any, is most likely not due to its enzymatic composition. We propose that it may rather be linked to the change in viscosity of the bolus, which would limit and/or slow down access of pepsin to the substrates.

To conclude, the use of human saliva instead of water had no substantial impact on protein hydrolysis. More importantly, comparison of the results found in this study on protein hydrolysis and values in the literature supports that preparation of a food bolus with realistic particle sizes is not a deterrent to the acquisition of reliable data using the pH-stat method. This methodological result opens the way to the use of the pH stat method to studies investigating the impact of oral physiology, in particular mastication, on digestion.

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CRediT authorship contribution statement

Martine Morzel: Conceptualization, Formal analysis, Writing – original draft. **Sylvaine Ramsamy:** Methodology, Investigation, Visualization. **Steven Le Feunteun:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Funding acquisition.

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.

References

Adler-Nissen, J., Eriksen, S., & Olsen, H. S. (1983). Improvement of the functionality of vegetable proteins by controlled enzymatic hydrolysis. *Plant Foods for Human Nutrition, 32*, 411–423. https://doi.org/10.1007/BF01091198

Bardow, A., Moe, D., Nyvad, B., & Nauntofte, B. (2000). The buffer capacity and buffer systems of human whole saliva measured without loss of CO2. *Archives of Oral Biology, 45*, 1–12. https://doi.org/10.1016/S0003-9969(99)00119-3

Baron, A., DeCarlo, A., & Featherstone, J. (2008). Functional aspects of the human salivary cystatins in the oral environment. *Oral Diseases, 5*, 234–240. https://doi.org/10.1111/j.1601-0825.1999.tb00307.x

Bo, L.-Y., Pang, J.-N., Song, C.-L., & Li, T.-J. (2019). Effect of the Plastein Reaction in Presence of Extrinsic Amino Acids on the Protective Activity of Casein Hydrolysate against Ethanol-Induced Damage in HHL-5 Cells. *Foods, 8*, 112. https://doi.org/10.3390/foods8040112

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*. https://doi.org/10.1038/s41596-018-0119-1

Church, F. C., Porter, D. H., Catignani, G. L., & Swaisgood, H. E. (1985). An o-phthalaldehyde spectrophotometric assay for proteinases. *Analytical Biochemistry, 146*, 343–348. https://doi.org/10.1016/0003-2697(85)90549-4

Deng, R., Seimys, A., Mars, M., Janssen, A. E. M., & Smeets, P. A. M. (2022). Monitoring pH and whey protein digestion by TD-NMR and MRI in a novel semi-dynamic in vitro gastric simulator (MR-GAS). *Food Hydrocolloids, 125*, Article 107393. https://doi.org/10.1016/j.foodhyd.2021.107393

Fang, X., Rioux, L.-E., Labrie, S., & Turgeon, S. L. (2016). Disintegration and nutrients release from cheese with different textural properties during in vitro digestion. *Food Research International, 88*, 276–283. https://doi.org/10.1016/j.foodres.2016.04.008

Grassl, N., Kulak, N. A., Pichler, G., Geyer, P. E., Jung, J., Schubert, S., … Mann, M. (2016). Ultra-deep and quantitative saliva proteome reveals dynamics of the oral microbiome. *Genome Medicine, 8*, 44. https://doi.org/10.1186/s13073-016-0293-0

Grundy, M.-M.-L., Edwards, C. H., Mackie, A. R., Gidley, M. J., Butterworth, P. J., & Ellis, P. R. (2016). Re-evaluation of the mechanisms of dietary fibre and implications for macronutrient bioaccessibility, digestion and postprandial metabolism. *British Journal of Nutrition, 116*, 816–833. https://doi.org/10.1017/S0007114516002610

Jalabert-Malbos, M.-L., Mishellany-Dutour, A., Woda, A., & Peyron, M.-A. (2007). Particle size distribution in the food bolus after mastication of natural foods. *Food Quality and Preference, 18*, 803–812. https://doi.org/10.1016/j.foodqual.2007.01.010

Li, Y., Hu, M., & McClements, D. J. (2011). Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: Proposal for a standardised pH-stat method. *Food Chemistry, 126*, 498–505. https://doi.org/10.1016/j.foodchem.2010.11.027

Luo, Q., Boom, R. M., & Janssen, A. E. M. (2015). Digestion of protein and protein gels in simulated gastric environment. *LWT - Food Science and Technology, 63*, 161–168. https://doi.org/10.1016/j.lwt.2015.03.087

Mat, D. J. L., Cattenoz, T., Souchon, I., Michon, C., & Le Feunteun, S. (2018). Monitoring protein hydrolysis by pepsin using pH-stat: In vitro gastric digestions in static and dynamic pH conditions. *Food Chemistry, 239*, 268–275. https://doi.org/10.1016/j.foodchem.2017.06.115

Mat, D. J. L., Souchon, I., Michon, C., & Le Feunteun, S. (2020). Gastro-intestinal in vitro digestions of protein emulsions monitored by pH-stat: Influence of structural properties and interplay between proteolysis and lipolysis. *Food Chemistry, 311*, 125946. https://doi.org/10.1016/j.foodchem.2019.125946

Mennah-Govela, Y. A., Keppler, S., Januzzi-Guerreiro, F., Follador-Lemos, C., Vilpont, K., & Bornhorst, G. M. (2020). Acid and Moisture Uptake into Red Beets during in Vitro Gastric Digestion as Influenced by Gastric pH. *Food Biophysics, 15*, 261–272. https://doi.org/10.1007/s11483-019-09623-w

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food & Function, 5*, 1113–1124. https://doi.org/10.1039/C3FO60702J

Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J., & Mattinen, M.-L. (2011). Effect of enzymatic cross-linking of β-casein on proteolysis by pepsin. *Food Hydrocolloids, 25*, 71–81. https://doi.org/10.1016/j.foodhyd.2010.05.007

Mulet-Cabero, A.-I., Egger, L., Portmann, R., Ménard, O., Marze, S., Minekus, M., … Mackie, A. (2020). A standardised semi-dynamic *in vitro* digestion method suitable for food – an international consensus. *Food & Function, 11*, 1702–1720. https://doi.org/10.1039/C9FO01293A

Neyraud, E., Bult, J. H. F., & Dransfield, E. (2009). Continuous analysis of parotid saliva during resting and short-duration simulated chewing. *Archives of Oral Biology, 54*, 449–456. https://doi.org/10.1016/j.archoralbio.2009.01.005

Repoux, M., Labouré, H., Courcoux, P., Andriot, I., Sémon, É., Yven, C., … Guichard, E. (2012). Combined effect of cheese characteristics and food oral processing on *in vivo* aroma release: *In vivo* aroma release during cheeses oral processing. *Flavour and Fragrance Journal, 27*, 414–423. https://doi.org/10.1002/ffj.3110

Salelles, L., Floury, J., & Le Feunteun, S. (2021). Pepsin activity as a function of pH and digestion time on caseins and egg white proteins under static *in vitro* conditions. *Food & Function, 12*, 12468–12478. https://doi.org/10.1039/D1FO02453A

Sheng, B., Nielsen, S. D., Glantz, M., Paulsson, M., Poulsen, N. A., & Larsen, L. B. (2022). Effects of genetic variants and sialylation on in vitro digestibility of purified κ-casein. *Journal of Dairy Science, 105*, 2803–2814. https://doi.org/10.3168/jds.2021-21289

Sun, X., Salih, E., Oppenheim, F. G., & Helmerhorst, E. J. (2009). Activity-based mass spectrometric characterization of proteases and inhibitors in human saliva. *Proteomics Clinical Applications, 3*, 810–820. https://doi.org/10.1002/prca.200800242

Thomadaki, K., Helmerhorst, E. J., Tian, N., Sun, X., Siqueira, W. L., Walt, D. R., & Oppenheim, F. G. (2011). Whole-saliva Proteolysis and Its Impact on Salivary

Diagnostics. *Journal of Dental Research, 90*, 1325–1330. https://doi.org/10.1177/0022034511420721

Tran Do, D. H., & Kong, F. (2018). Texture changes and protein hydrolysis in different cheeses under simulated gastric environment. *LWT - Food Science and Technology, 93*, 197–203. https://doi.org/10.1016/j.lwt.2018.03.028

Zhao, J., Wu, J., Chen, Y., Zhao, M., & Sun, W. (2020). Gel Properties of Soy Protein Isolate Modified by Lipoxygenase-Catalyzed Linoleic Acid Oxidation and Their Influence on Pepsin Diffusion and In Vitro Gastric Digestion. *Journal of Agricultural and Food Chemistry, 68*, 5691–5698. https://doi.org/10.1021/acs.jafc.0c00808