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From static to semi-dynamic *in vitro* digestion conditions relevant for the older population: starch and protein digestion of cooked lentils†

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In the context of adequately feeding the rising older population, lentils have an important potential as sources of (plant-based) protein as well as slowly digestible bio-encapsulated starch and fibre. This study evaluated *in vitro* digestion of protein and starch in lentils under conditions representing the gastrointestinal tract of older adults. Both static and semi-dynamic simulations were applied to analyze the effect of specific gastrointestinal conditions (healthy *versus* older adult) on macronutrient digestion patterns. Gastric proteolysis was strongly dependent on applied gastric pH (gradient), leading to a lower extent of protein hydrolysis for simulations relevant for older adults. Fewer and smaller (lower degree of polymerization, DP) bioaccessible peptides were formed during gastric proteolysis under older adult compared to healthy adult conditions. These differences, developed during the *in vitro* gastric phase, were compensated during small intestinal digestion, yielding similar final proteolysis levels regardless of the applied simulation conditions. In contrast, in the presence of saliva, amylolysis was generally accelerated under older adult conditions. Moreover, the current work highlighted the importance of considering saliva (or salivary amylase) incorporation in simulations where the applied gastric pH (gradient) allows salivary amylase activity. Under both healthy and older adult conditions, *in vitro* starch hydrolysis bio-encapsulated in cotyledon cells of cooked lentils was attenuated, compared to a white bread reference.

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1 Introduction

The population of older adults (defined as over 65 years of age) is growing and projected to reach 12% of by 2030.[1] This group is very heterogeneous in terms of activity level and (chronic) disease burden. Aging goes along with several physiological changes throughout the gastrointestinal tract. Changes in the oral cavity include (but are not limited to) loss of dentition, decrease in salivary secretion, and lowered sensorial perception and appetite.[2,3] Gastric changes include a suppressed gastric acid secretion, as well as slower stomach mobility and gastric emptying.[3] With aging, a decrease in pancreatin and bile secretions were reported in the small intestine, as well as a decreased intestinal motility.[3–5]

Due to these gastrointestinal changes, social, psychological, and economic factors, aging is typically linked with a decreased food intake (around 25%),[3] weight loss, and the risk of developing malnutrition and sarcopenia (the loss of muscle mass and function).[4–6] Due to metabolic changes, older adults require a higher protein intake to maintain muscle mass compared to healthy adults. Therefore, while older adults have a lower caloric requirement, ingestion of 1–1.5 g protein per kg body mass per day (in combination with physical exercise) is recommended in comparison to the 0.8 g kg^{-1} day^{-1} recommended for healthy adults.[6,7] Additionally, aging is associated to a decrease in insulin secretion and action, causing decreased glucose tolerance.[8,9] Maintenance of proper glycaemic control through a healthy diet, rich in fibre and low GI (glycaemic index) foods[10] is of importance for both management and prevention of type 2 diabetes, in particular for older adults.[11]

It is clear that adapted nutrition is of crucial importance for the aging population. Therefore, research on the impact of gastrointestinal changes occurring with aging on carbohydrate and protein digestion is necessary. Due to the GI changes stated above, (*in vitro*) digestion models designed for healthy adults are not suitable to mimic digestion processes in older adults.[12] Therefore, *in vitro* digestion models simulating gastrointestinal conditions of the older population are increasingly being developed and applied.[5,13–17]

Pulses are healthy and sustainable wholefoods, rich in protein, slowly digestible starch, fibre, vitamins, and minerals.

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Upon cooking of pulses followed by mechanical disintegration (such as chewing), pulse cells remain intact, encapsulating dietary protein and starch.[18,19] Due to the barrier function of the intact cell wall, cooked pulses have been linked to slower starch digestibility *in vitro*,[20–22] and slower postprandial glucose increase glycaemic index (GI) *in vivo*.[23] Additionally, encapsulation of protein inside cooked pulse cells has also been shown to slow proteolysis *in vitro*,[20,24,25] but increase the concentration of serum amino acids *in vivo*.[26] Potentially, pulses such as lentils could be an important nutrient source for older adults. While a recent study revealed slowed proteolysis of a pea protein suspension under static older compared to healthy adult *in vitro* digestion conditions,[27] digestion of co-ingested starch and protein present within (cotyledon cells of) whole cooked pulses, has not been studied under (*in vitro*) conditions relevant for the older population until now.

The aim of this work was threefold. Firstly, starch and protein digestion kinetics of cooked lentils were studied under *in vitro* conditions relevant for older adults. Doing this, it could be evaluated to which level typical attenuated hydrolysis patterns of intact cotyledon cells (such as slow starch digestion), observed using healthy adult *in vitro* models, were affected by gastrointestinal conditions observed in older adults. White bread was selected as a reference food system since it is a well-studied source of both (rapidly digestible) starch and protein,[27–29] and it is much-consumed by the targeted population group. The comparison of lentil digestion kinetics to those of white bread could deliver insight into the (possibly different) consequences of applying altered digestion conditions for two differently structured foods. Secondly, static up to more advanced semi-dynamic approaches were combined to obtain further insight on how those dynamically changing digestion conditions (relevant for healthy *versus* older adults) affect digestion kinetics, and how semi-dynamic digestion kinetics compare to their static counterparts. Thirdly, the importance of incorporating salivary amylase was evaluated in the context of digestion conditions relevant for the older population. Saliva (with salivary amylase) is often omitted from *in vitro* digestion research (focusing on healthy adults) due to its high cost and limited expected contribution to overall amylolysis.[12] However, orogastric amylolysis was expected to be significant in simulations allowing higher enzyme activity (*i.e.*, limited or gradual gastric acidification).[30,31]

2 Materials & methods

2.1 Materials

Digestion enzymes (pepsin, pancreatin, trypsin, and chymotrypsin) and most chemical reagents were purchased from Merck (Belgium). Pooled human saliva was obtained from Lee Biosolutions (Maryland Heights, MO, USA). The Total Starch Kit was obtained from Megazyme (Bray, Ireland). All reagents were of analytical or HPLC grade.

Du Puy lentils (Canada, 2019) were donated by Casibeans, sorted, and stored at −40 °C (below the glass transition temperature at ~10% moisture) until processing. Raw lentils were soaked, then cooked to palatable texture for 30 min at 95 °C, in similarity to a previous study.[32] White bread was obtained from the local supermarket and utilized as provided.

2.2 Static *in vitro* digestion

For healthy adult simulations, static *in vitro* digestion was carried out using to the standardized INFOGEST 2.0 protocol,[33] as summarized in section 2.3.1 and described in detail earlier.[32] Standardized static simulation conditions were varied to parameters relevant for the older adult population, as explained in section 2.3.2.

2.2.1 Static *in vitro* digestion protocol for healthy adults

Oral phase. Both cooked lentils and white bread are solid foods which require disintegration before ingestion, for example by mastication or by mechanical means (mixing). Since this work aims to study the impact of specific gastrointestinal conditions (reported to change upon aging) on macronutrient digestion kinetics, the mechanical disintegration step was standardized as recommended in the semi-dynamic INFOGEST protocol,[34] and digestion simulations were carried out using 'standardized purees'. These purees were produced in order to represent a bolus as realistically as possible both in terms of *(i)* consistency and *(ii)* microstructural distribution. In terms of consistency, the ratio of simulated salivary fluid SSF (80% electrolyte simulated salivary fluid (pH 7), 0.5% $CaCl_2$ (0.3 M), and 19.5% demineralized water) and cooked lentils and bread was determined, yielding relevant bolus consistencies as proposed in the standardized INFOGEST protocol.[34] For lentils, a final ratio of food : SSF of 1 : 2 (mass : volume) was selected. To align the samples with regards to a realistic bolus consistency,[34] a different ratio of 1 : 3 was selected for white bread. Regarding the microstructure, particle size distributions (PSDs) of cooked lentils, mixed at low speed in SSF were shown to largely overlap with PSDs of cooked beans upon *in vivo* mastication in previous work.[19,32] Therefore, lentils and white bread were mixed into standardized purees using an Ultraturrax mixer (3000 rpm).

To investigate the effect of saliva (and salivary amylase) on gastrointestinal (starch) digestion, simulations were carried out both with and without saliva. The amount of saliva required to reach 150 U mL^{-1} SSF was calculated upon analysis of the amylase activity of human pooled saliva.[33] This volume was omitted from the food : SSF mixture during puree mixing. In this manner, the appropriate amount of saliva could be added at the initiation of each digestion experiment, finally reaching the required bolus consistency and salivary amylase activity. For simulations without amylase, this volume was replaced with SSF.

Gastric phase. After the oral phase, simulated gastric fluid (SGF, pH 3) and $CaCl_2$ (0.3 M) were added, after which the pH was controlled at 3 (see Table 1). Water and porcine pepsin solution were added, obtaining a final volume of 5 mL with a 2000 U mL^{-1} pepsin activity. Digestion tubes were incubated (37 °C) for 2 h, under head-over-heels rotation (70 rpm). Independent samples were taken at 0, 5, 10, 20, 30, 45, 60, 90,

Table 1 Applied static and semi-dynamic *in vitro* digestion conditions, relevant for healthy adults and older adults in this study

Oral phase	Healthy adult		Older adults	
	No saliva or saliva (150 U mL^{-1} SSF salivary amylase)[38]		No saliva or saliva (150 U mL^{-1} SSF salivary amylase)[16,39,40]	
	Static simulation[33]	Semi-dynamic simulation[38,41]	Static simulation	Semi-dynamic simulation
Gastric phase	2000 U mL^{-1} pepsin Static pH 3 2 hours	Gradual addition of 2000 U mL^{-1} pepsin Gradual acidification to pH 2 2 hours	1500 U mL^{-1} pepsin[13,14,42] Static pH 6[13,42] 4 hours[13]	Gradual addition of 1500 U mL^{-1} pepsin[14] Gradual acidification to pH 4[5,17,43] 2 hours
Small intestinal phase	Bile 10 mM 200 U mL^{-1} α-amylase 100 U mL^{-1} trypsin 25 U mL^{-1} chymotrypsin pH 7 2 hours[33,38]		5 mM bile[5] 100 U mL^{-1} α-amylase[5,13] 50 U mL^{-1} trypsin[5,13] 12,5 U mL^{-1} chymotrypsin[5,13] pH 7 4 hours (static)[14] or 2 hours (semi-dynamic)	

SSF: simulated salivary fluid.

and 120 min, followed by thermal enzyme inactivation (5 min, 98 °C).[18,19,35,36]

Small intestinal phase. Simulated intestinal fluid (SIF, pH 7), $CaCl_2$ (0.3 M) and fresh bile solution were added to each digestion tube, and the pH was brought to 7. Finally, demineralized water and pancreatic enzyme solution (in SIF) were added to each tube, reaching a bile concentration and enzyme activities as prescribed in the INFOGEST protocol[33] and indicated in Table 1. Digestion tubes were incubated, and enzymes were thermally inactivated for individual samples at 0, 5, 10, 20, 30, 45, 60, 90, and 120 min. Digestion tubes were centrifuged (5 min, 2000*g*, Sigma 4–16 KS, Sigma, Osterode am Harz, Germany), to allow separation of the soluble metabolites in the supernatant, and the non-bioaccessible, insoluble pellet, as previously reported.[19,37] Thereafter, supernatants were snap frozen in liquid nitrogen, and stored at −40 °C until further analysis.

2.2.2 Adaptations to the static *in vitro* digestion protocol for the conditions of older adults

Oral phase. The aim of this work was to evaluate the effect of (altered) static and semi-dynamic gastrointestinal conditions, relevant for healthy adults *versus* older adults, on starch and protein digestibility. While the authors reckon that changes in oral conditions with aging (loss of teeth, decreased production of saliva *etc*.) can significantly impact bolus formation and subsequent digestion and nutrient uptake,[4,28] this was not the major focus of the current work. Therefore, identical standardized lentil purees were used for simulating digestion under conditions relevant for older and healthy adults, as explained earlier.

Literature on oral conditions of older adults reveals that this group typically produces a lower amount (40–50%[39,40]) of more concentrated saliva (up to double amylase activity in saliva of elderly compared to healthy adults[44,45]). Considering the focus of this study and the choice for a constant dilution of the food (*cfr.* standardized puree), an identical amount of saliva was applied during the oral phase regardless of targeted aged group (and thus identical salivary amylase activity, Table 1). Similarly, no difference in salivary amylase activity per mL SSF compared to healthy adult model was proposed in the recently published standardized INFOGEST protocol for older adults.[16]

Gastric phase. In older adults, generally, lower pepsin release and (s)lower acidification are observed in the stomach.[4,5] These conditions were therefore adapted in the older adult model.

A pepsin activity decrease of ~25% was reported in the older compared to the healthy adult population.[5,46] Accordingly, the applied pepsin activity during the (static) simulated gastric phase was decreased to 1500 U mL^{-1} (Table 1), in accordance with previously published static elderly models.[13,14,42] Higher fasted pH values and a (s)lower acidification were generally observed in older compared to healthy adults (6.2 to 2 *versus* 4.5 to 1.2), as reviewed by Shani Levi & Lesmes (2014).[5] In more detail, heterogeneous gastric pH profiles were observed *in vivo*, ranging from a low fasted pH with a postprandial pH decrease, to an elevated fasted pH with a very limited acidification and high postprandial pH.[43] Due to the heterogeneity in gastric pH profiles, it is impossible to capture the whole older population by one best guess average gastric pH. While the first group has a gastric pH profile similar to those found in healthy adults,[47] the gastric pH of the second group is generally high (not dropping below 5.5 postprandially[43]). This group encompasses people with hypo- and achlorhydria, *i.e.*, the limited release or absence of gastric acid secretion in the stomach, with an increased prevalence in the older population.[2] Therefore, a static gastric pH of 6 was selected for this part of the (static simulation) work (Table 1), in accordance with conditions previously defined for static elderly digestion model.[13,42] While it is an extreme condition, this pH value of 6 relevantly represents the subpopulation with the most limited gastric acidification,[42] as well as (a large group of older) people taking proton pump inhibitors (PPI) for an array of gastric acid-related conditions. Additionally, comparison of digestion kinetics obtained under high pH conditions *versus* kinetics obtained applying the

healthy adult model (at gastric pH 3), can document the range between which digestion kinetics relevant for older adults could be expected. However, a more realistic approach could be the implementation of gradual gastric pH profiles (adapted to the healthy or older adult population), as discussed in section 2.3.

Small intestinal phase. With aging, decreases in both pancreatic enzyme and bile secretions were reported.[4,5] In accordance with *in vivo* data,[43] and conditions previously selected for older adult *in vitro* models,[5,13] both the bile concentration and activities of pancreatic enzymes were reduced by 50% (Table 1). To mimic the longer transit time reported for older adults, small intestinal incubation was increased to 4 h.[13] Apart from these changes, the small intestinal simulation was carried out as explained in section 2.2.1, with additional sampling points at 150, 180, and 240 minutes.

2.3 Semi-dynamic *in vitro* digestion

Using semi-dynamic models, it is possible to study the effect of dynamically changing parameters on nutrient digestion kinetics.[12] Since the main focus of this work is studying digestibility of lentil macronutrients, these more complex simulations were only carried out for lentil purees (section 2.2.1). In accordance with the proposed semi-dynamic INFOGEST protocol,[34] the effect of gradually changing gastric conditions was evaluated (*i.e.*, pH and pepsin activity), while the small intestinal phase was simulated in a static manner (*i.e.*, exactly as explained in section 2.2).

To simulate semi-dynamic orogastric digestion, a custom-tailored multireactor digestion system (MuReDi, BioXplorer 100, H.E.L. Group) was employed, introduced in a recent study by our research unit.[41] The MuReDi system has 8 independent reactors, one for each analysed digestion time point. With this equipment, digestive fluids can be gradually added throughout digestion, using separate syringe pumps connected to each reactor, while the reactors are continuously stirred (250 rpm) and monitored in terms of temperature and pH. The MuReDi system is ran by a computer-controlled software (WinISO) in which commandos can be entered to program the digestion experiment in a stepwise manner.[41]

The composition and pH of the gastric solution (containing SGF, $CaCl_2$, HCl and H_2O) was determined in a preliminary experiment, so that, upon addition of the complete volume of gastric and pepsin solutions, a final pH of 2 (healthy adult simulations) or 4 (older adult simulations) were reached (see Table 1). Therefore, a gastric solution with a pH of 1.02 was used for healthy adult simulations, while this was pH 1.45 for older adult simulations. On the day of the digestion experiment, gastric and pepsin solutions were made fresh. Both solutions were linked to the inlet lines of separate syringe pumps of the MuReDi system, and the pump and reactor feed lines were primed. Before the start of each digestion simulation, lentil puree (see section 2.2.1) was weighed into each of the 8 reactors. Each reactor was connected to the MuReDi system, pump lines were connected, and the prewritten program was launched.

Oral phase. Firstly, the lentil samples were heated to 37 °C under constant stirring (250 rpm). After exactly 6 min, the oral phase was launched upon adding a predetermined amount of saliva, reaching 150 U salivary amylase per mL SSF and a total volume of 30 mL (containing 10 g of cooked lentils, as explained in section 2.2.1). For experiments not considering saliva, this volume was replaced by SSF (exactly as carried out for static simulations, section 2.2.1). Samples were incubated for 2 minutes under constant stirring and heating (37 °C).

Gastric phase. A basal volume of 10% of the total gastric and pepsin solutions was pumped into each of the reactors, and the gastric phase was initiated. Over the course of 120 min of gastric incubation, the remaining 90% of both fluids were gradually added into each reactor. For healthy adult simulations, the rates of pepsin and gastric fluid pumping were set, so that a complete volume of 60 mL, a pH of 2, and a pepsin activity of 2000 U mL^{-1} were reached after 2 hours of gastric digestion. For older adult simulations, a pH of 4 and final pepsin activity of 1500 U mL^{-1} were targeted. For sampling during the gastric phase, independent reactors were stopped at different predetermined gastric digestion times, *i.e.*, 5, 10, 20, 30, 45, 60, 90, and 120 minutes. At these time points, the contents of the reactor were homogenously sampled into glass test tubes, and thermally inactivated. In contrast, when studying the digestion phenomena during the small intestinal phase, all reactors underwent 2 h of gastric digestion simulation, reaching a final volume of 60 mL, before initiating small intestinal simulations. The gastric pH profiles obtained during the gastric phase are reported in Appendix A.

Small intestinal phase. After completion of the gastric phase, a small intestinal fluid mixture (containing SIF, $CaCl_2$ and fresh bile solution) was instantly added to each of the reactors, immediately increasing the pH to values of 6.5–7. When necessary, the pH was further increased to 7 using NaOH (2 M). To initiate the static small intestinal phase, a pancreatin solution was added to each of the reactors, reaching a final volume of 120 mL, a pH of 7, and bile concentration and pancreatic enzyme activity as indicated in Table 1 for healthy and older adult simulations. While static *in vitro* small intestinal digestion was followed for 4 hours (Table 1), preliminary data of static digestion experiments under older adult conditions showed that macronutrient hydrolysis levelled off after around 120 min. Therefore, semi dynamic digestion simulations were evaluated for 2 h, with independent reactors stopped at 5, 10, 20, 30, 45, 60, 90, and 120 min. The reactor contents were then transferred to a glass tube and enzymes were thermally inactivated. Then, supernatant and pellet were separated upon centrifugation (5 min, 2000*g*) and stored (−40 °C) until analysis.

2.4 Quantification of macronutrient digestion

2.4.1 Compositional analysis. The dry matter content of the cooked lentils and white bread were determined upon duplicate oven drying. To analyse the starch and protein content, whole cooked lentils and white bread were lyophilized (Alpha 1–4, LSCplus, Martin Christ, Germany), followed by

pounding in a ball mill (MM400, Retsch, Haan, Germany). The Total Starch Kit (AA/AMG, Megazyme Inc. Bray, Ireland) was employed to determine the starch content in duplicate. Sample nitrogen was quantified in duplicate using automated Dumas analysis (CHNS-O Elemental Analyzer, CE instrument, Thermo Fischer Scientific, Waltham, MA, USA), after which the crude protein content was calculated employing 5.4 as specific conversion factors for pulses and wheat.[48]

2.4.2 Quantification of digested protein. Proteolysis was quantified during the gastric and small intestinal phases using the spectrophotometric *o*-phtaldialdehyde (OPA) method,[49,50] exactly as described previously.[20] While more in-depth methods such as size exclusion chromatography exist to study proteolysis and peptide release patterns during digestion, OPA analysis remains a much applied and straightforward manner to screen protein digestibility. Moreover, results obtained by OPA analysis were highly correlated to those obtained with more advanced chromatographic methods, supporting its suitability for digestibility screening.[37]

In brief, the total amount of α-amino groups in the undigested food (lentil or bread) sample (NH_{2total}) was determined by hydrolysing undigested sample proteins to amino acid constituents using strong acid (6 N HCl, 24 h, 110 °C). The amount of terminal amino groups was then determined spectrophotometrically, employing the OPA-reagent and a L-serine standard curve (12.5–100 mg mL^{-1}).

Then, the extent of proteolysis was determined in the digestive supernatant and expressed relative to NH_{2total}. To do so, large peptides and proteins were precipitated from the digestion supernatant upon addition of 3.2% TCA, yielding the *readily bioaccessible fraction* (NH_{2TCA}).[50,51] This fraction is considered readily bioaccessible as such (without additional hydrolysis by brush border enzymes).[30] The terminal amino groups of this fraction were quantified using OPA. This analysis only quantifies the increase in small peptides upon enzymatic hydrolysis of proteins and peptides but does not take into account larger peptides formed. Additionally, no distinction is made between peptides with different polymerization degrees (DPs).[37] Therefore, in some cases, proteolysis was quantified in more absolute terms, by hydrolysing the readily bioaccessible peptide fraction into constituting amino acids, yielding *the hydrolysed readily bioaccessible fraction* ($NH_{2TCA,hydro}$).[30] Blanks (containing all reagents and enzymes but no sample) were used to correct for the protein originating from the enzyme solutions. From the ratio of $NH_{2TCA,hydro}$ and NH_{2TCA}, an average polymerization degree (DP) of the readily bioaccessible peptide fraction could be calculated.[20]

2.4.3 Quantification of digested starch. Since salivary amylase was incorporated in certain digestion simulations, starch digestion was evaluated for each studied digestion time during both the gastric and small intestinal phases. Digested starch was quantified spectrophotometrically using the dinitrosalicylic (DNS) method, employing a maltose calibration curve (0.5–2.0 mg mL^{-1}), exactly as described earlier.[32]

2.5 Statistical data analysis and modelling

For each (static or semi-dynamic) simulation, kinetics of starch and protein digestion were analysed using an independent reactor for each individual considered timepoint. This means that each digestion tube/reactor represents an independent evaluation of the digestion kinetics of a food system, at different time points during *in vitro* digestion, and the data can be analysed together.[12] Therefore, the experimental data obtained over at least 8 independent experimental timepoints were integrated using an empirical first order model, showing good fit of the data. In this case, as was the case for previous static and semi-dynamic *in vitro* digestion studies of our research unit,[30,41] a fractional conversion model was selected (shown in eqn (1)).

$$C(t) = \boldsymbol{C_f} + (C_0 - \boldsymbol{C_f}) \times e^{-\boldsymbol{k} \times t} \quad (1)$$

C (%) is the extent of starch or protein hydrolysis at a certain digestion time, while C_0 (%) is defined as the extent of hydrolysis at the beginning of the (small intestinal or gastric) digestion phase. Nonlinear regression (SAS version 9.4, SAS Institute, Inc., Cary, NC, USA) was employed to simultaneously estimate $\boldsymbol{C_f}$ (%), the final hydrolysis extent, and $\boldsymbol{k}$ (min^{-1}), the rate constant of the hydrolysis. The fit of the model was evaluated using residue and parity plots, and by calculation of R^2_{adj}. Since $\boldsymbol{k}$ and $\boldsymbol{C_f}$ were estimated based on consecutive and independent evaluations of the same system (one tube/reactor per tested digestion time), the standard error of those parameters reflects the uncertainty of independent measurement of the digestion extent of the same food system (at different time points). Then, modelled curves were compared and 95% confidence intervals were used to verify significant differences between estimated parameters $\boldsymbol{k}$ and $\boldsymbol{C_f}$.

3 Results & discussion

3.1 Chemical composition

Cooked lentils had a starch and protein content of 48.7 ± 0.4% and 21.4 ± 0.1% (dry matter), respectively. The white bread contained 59.6 ± 1.4% starch and 9.6 ± 0.1% protein on dry matter basis. The dry matter content of cooked lentils and bread were 25.5 ± 0.1%and 62.5 ± 0.4%, respectively.

3.2 Static *in vitro* digestion using digestion models relevant for healthy adults *versus* older adults

3.21 Protein digestion. Gastric and small intestinal proteolysis are shown in Fig. 1, for both foods digested *in vitro* under healthy and older adult static digestion models, with and without saliva. Data showing a rapid initial increase, followed by a gradual levelling off, were modelled using the fractional conversion model (eqn (1)), with estimated kinetic parameters reported in Table 2.

Gastric proteolysis occurred faster and to a higher extent during simulations under healthy adult (~5–7%) *versus* older adult (~1–2.5%) conditions, regardless of the considered food

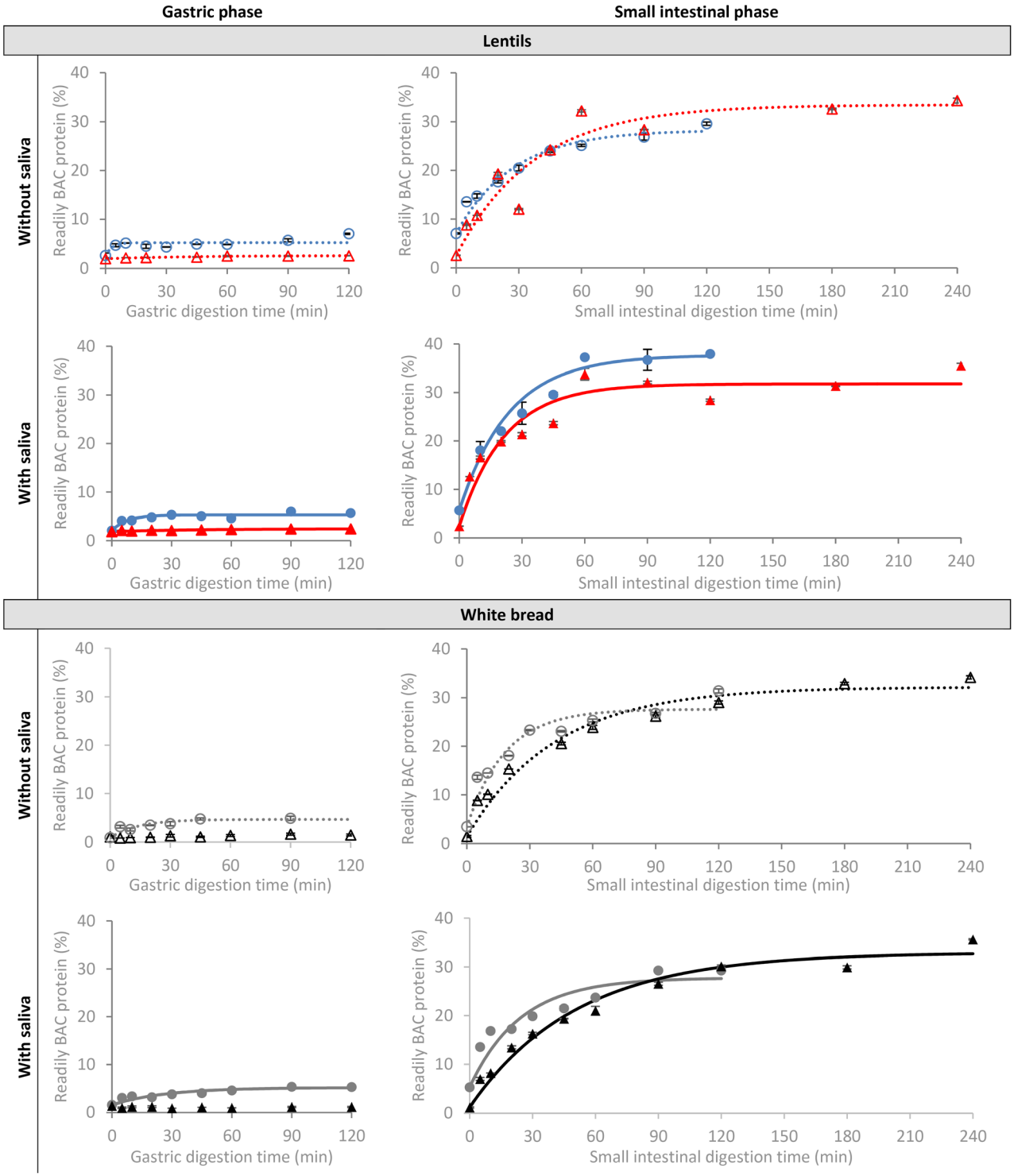

Fig. 1 Protein hydrolysis during gastric and small intestinal *in vitro* digestion of cooked lentils and white bread, applying static *in vitro* models relevant for healthy adults (○/● and ○/●, for lentils and bread, resp.) and older adults (△/▲ and △/▲ for lentils and bread, resp.). Open symbols indicate simulations without addition of saliva in the oral phase, while full symbols represent simulations including saliva. The standard deviation of analytical replicates is indicated by error bars.

type or saliva addition. Generally, low levels of gastric proteolysis (in terms of % readily bioaccessible protein) can be explained by the quantification method applied, only measuring TCA-soluble small peptides, limitedly formed during (initial) gastric pepsinolysis. A (s)lower gastric proteolysis was expected under older adult conditions, based on the lower dosage of pepsin and the relatively high gastric pH causing a low pepsin activity (*i.e.*, ~15 times reduction of peptic activity

Table 2 Estimated kinetic parameters (±standard error) estimated using the fractional conversion model (eqn (1)) for the formation of readily bioaccessible (BAC) protein during gastric and small intestinal static *in vitro* digestion applying models relevant for healthy and older adults. The rate constant k and final extent of hydrolysis C_f are reported, with different letters within a column indicating a significant difference between means based on 95% confidence intervals

		Gastric phase			Small intestinal phase		
		k (min^{-1})	C_f (%)	R^2_{adj}	k (min^{-1})	C_f (%)	R^2_{adj}
			Lentil				
No saliva	Healthy adult	0.309 ± 0.306^{a}	5.3 ± 0.3^{a}	0.97	$0.037 \pm 0.006^{a,b}$	28.3 ± 1.1^{b}	0.99
	Older adult	0.023 ± 0.006^{a}	2.6 ± 0.1^{b}	0.99	$0.027 \pm 0.006^{a,b}$	$33.5 \pm 2.5^{a,b}$	0.97
Saliva	Healthy adult	$0.130\ 0.041^{a}$	5.3 ± 0.2^{a}	0.99	$0.042 \pm 0.007^{a,b}$	37.7 ± 2.0^{a}	0.99
	Older adult	0.022 ± 0.008^{a}	2.5 ± 0.1^{b}	0.99	$0.046 \pm 0.010^{a,b}$	$31.8 \pm 1.6^{a,b}$	0.98
			White bread				
No saliva	Healthy adult	0.072 ± 0.027^{a}	4.7 ± 0.5^{a}	0.97	0.055 ± 0.012^{a}	27.6 ± 1.6^{b}	0.98
	Older adult	n.a.	n.a.	n.a.	$0.024 \pm 0.004^{a,b}$	$32.2 \pm 1.5^{a,b}$	0.99
Saliva	Healthy adult	0.037 ± 0.011^{a}	5.2 ± 0.4^{a}	0.98	$0.041 \pm 0.002^{a,b}$	27.8 ± 2.0^{b}	0.98
	Older adult	n.a.	n.a.	n.a.	0.020 ± 0.002^{b}	$32.9 \pm 1.4^{a,b}$	0.99

at pH 6 compared to 3[52]). Additionally, the pH during the gastric phase could affect the protein conformation,[53] and subsequently its susceptibility to enzymatic hydrolysis. In line with these results, a decrease in gastric proteolysis was observed in pea, whey, rice, soy and meat under gastric conditions relevant for elderly individuals.[27,54] In contrast, no significant decrease in gastric proteolysis was observed for wheat protein by Melchior *et al.* (2023).[27] This difference could potentially be explained by the difference in applied simulation conditions, *i.e.*, a gastric pH 3.7 compaared to the pH 6 used in this work (see Table 1).

As expected, saliva addition had no significant effect on the formation of readily bioaccessible peptides during the gastric phase. Additionally, though bread and lentil protein are very different in terms of composition and microstructural organization, no significant differences between bread and cooked lentils were detected in terms of gastric readily bioaccessible peptide formation.

During **small intestinal digestion**, differences in proteolysis extent present at the end of the gastric phase were gradually compensated. During the first 60 minutes of small intestinal digestion, the amount of readily bioaccessible protein remained higher for each analysed time point under the healthy adult compared to the older adult conditions. This could be explained by the differences caused by gastric predigestion as well as the higher enzyme activity applied during healthy adult simulation of the small intestinal phase. However, upon longer digestion times, proteolysis reached similar final values C_f, with no significant differences between healthy and older adult simulations.

Generally, saliva addition seemed to have a small (but non-significant) positive effect on the proteolysis degree (expressed as readily bioaccessible peptides) achieved by the end of the small intestinal phase. For the case of the static healthy adult simulation of lentil digestion upon saliva incorporation, however, a significantly higher final proteolysis level C_f was observed. In literature, proceeding degradation of starch (initiated during the oral phase upon addition of salivary amylase) has been described to gradually render proteins more accessible to proteolytic enzymes, resulting in a higher overall protein digestibility.[29,55,56] Additionally, the formation of readily bioaccessible protein could potentially be increased to some extent by the addition of saliva through precipitation of certain protein fractions (food protein aggregation and precipitation by salivary mucins have been reported through different interaction modes[57]), improving the accessibility of more soluble peptides to proteolytic enzymes.

These results are in line with final levels of proteolysis reported for digestion of bread, and wheat and pea protein under healthy *versus* older adult conditions (gastric pH 4.5).[27,29] In contrast, other studies observed a decreased small intestinal protein digestibility using an elderly model similar to the one employed here for different meat[14] and fish[13] samples, quantifying released amino acids using a GC-MS system. Possibly, a readily bioaccessible peptide fraction with different molecular weight or DP distribution could be formed as a result of digestion under healthy *versus* older adult conditions. In the above, terminal amino groups of small TCA-soluble peptides were quantified by OPA as a measure of proteolysis. While OPA is a suitable method for straightforward digestibility screening (see section 2.4.3), it does not allow for the absolute quantification or identification of these small peptides.[37] Therefore, additional quantification of readily bioaccessible small peptide fraction formed upon digestion was carried out, upon complete hydrolysis of small peptide fraction into its amino acid constituents. From these data in terms of amino acid constituents, the average DP of the readily bioaccessible fraction was calculated and shown in Fig. 2.

Gastric proteolysis under older adult conditions converted only 7–8% of the total protein into readily BAC peptides, with an average DP of around 3. In contrast, at the end of the healthy adult gastric phase, about 26–28% of protein was rendered readily bioaccessible, with an average DP of 4.5–5. As previously stated, this higher digestibility could be explained by the higher dosage and pH-dependent activity of pepsin in the healthy adult gastric phase (Table 1). By the end of the small intestinal simulation, however, this difference was compensated. Similar amounts of readily BAC peptides with

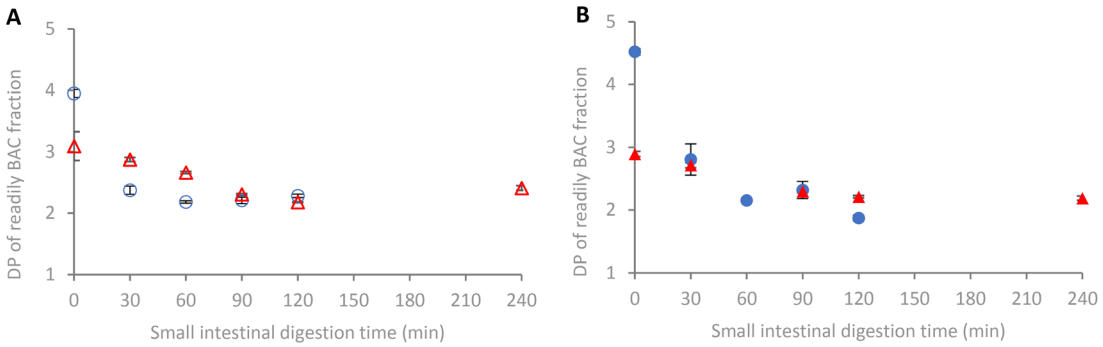

Fig. 2 Average polymerization degree (DP) of the readily bioaccessible (BAC) peptide fraction formed upon small intestinal proteolysis of cooked lentils, applying static *in vitro* models relevant for healthy adults (○/●) and older adults (△/▲), (A) for simulations without saliva (open symbols), and (B) for simulations with saliva (full symbols) The standard deviation of analytical replicates is indicated by error bars.

similar DPs were formed at the end of small intestinal simulation, *i.e.*, ~68–71% for the healthy adult (120 min, DP 1.9–2.2) and 78–83% for older adult models (240 min, DP 2.2–2.4). These findings support and clarify the previously discussed results in terms of terminal amino groups of the readily BAC fraction, showing (small but) significant differences between the healthy and older adult conditions during the gastric phase, which are compensated during the small intestinal phase (Table 2). However, the older adult simulation conditions do not only cause the formation of a lower amount of small peptides during gastric proteolysis, but the formed peptides are also smaller in terms of polymerization degree, compared to pepsinolysis under healthy adult conditions. These observations support that altered digestion conditions, such as those which occur upon aging, could affect proteolysis kinetics as well as peptide formation patterns during gastrointestinal digestion, even though similar final digestion extents might be reached. These proteolytic patterns have been linked to several physiological mechanisms such as satiation,[58] and should therefore be studied in more detail, with regards to adapting nutritional recommendations for population groups with altered gastrointestinal conditions.

3.2.1 Starch digestion. Amylolysis in function of static gastric and small intestinal digestion is shown in Fig. 3. Where relevant, data were fitted with a fractional conversion model (eqn (1)), with estimated model parameters reported in Table 3.

In absence of saliva, logically, no amylolysis took place during the simulated **gastric phase**. In contrast, in presence of saliva, the course of gastric amylolysis was strongly depending on the applied simulation conditions. Under static healthy adult conditions (applied to both bread and lentils), virtually no gastric starch hydrolysis by salivary amylase took place, due to its inactivation at pH 3.[31,59] In contrast, under higher pH conditions (older adult model at constant gastric pH 6), amylolysis in lentils reached ~65% after 2 h of gastric simulation. Although the model fits the data well, amylolysis in lentils was not yet significantly levelling off after 2 h. Therefore, C_f could not be accurately estimated and should be interpreted carefully. A similar amylolysis profile was obtained when only increasing the gastric pH of the healthy adult model (healthy adult model with increased gastric pH 6, *data not shown*), confirming the importance of the pH-dependent amylase activity for determining the amylolysis rate. For bread, gastric amylolysis by salivary amylase at pH 6 (older adult conditions) occurred much more rapidly (k significantly higher) compared to cooked lentils, reaching a clear plateau ~76% after ~30 min, explained by the encapsulation of lentil starch inside intact cotyledon cells.

Based on these results and in accordance with previously published findings,[31,60] orogastric starch digestion should not be overlooked in simulations where the gastric pH allows salivary amylase activity. Since a constant pH of 3 (or 6) in the stomach is not realistic for most of the adult population, the combination of incorporating both salivary amylase and more realistic (gradually decreasing) gastric pH profiles into digestion simulations, as discussed in section 3.3, can be of especially high importance for obtaining a better understanding of (more realistic) amylolysis patterns under conditions relevant for different population groups.

Small intestinal amylolysis extents of ~95–100% were obtained regardless of the applied simulation conditions. However, these applied conditions (healthy *versus* older adult model and saliva omission *versus* incorporation) significantly impacted the observed small intestinal amylolysis kinetics.

In the presence of saliva, for the case of lentils, the degree of amylolysis at the beginning of the small intestinal phase was much higher for the older (~65%) compared to the healthy adult (~0%) simulation. Consequently, a rapid amylolysis took place during the healthy adult small intestinal phase, compared to a more gradual amylolysis under the older adult conditions, though reaching similar final values. In accordance with the current study, a more rapid amylolysis was observed in white bread applying an older adult model (including salivary amylase) compared to a healthy adult model, however reaching similar final starch hydrolysis extents.[29]

In absence of salivary amylase, small intestinal amylolysis in lentils took place at a higher rate (significantly higher rate constant k) for the healthy compared to the older adult simulation. This could be explained by the higher pancreatic

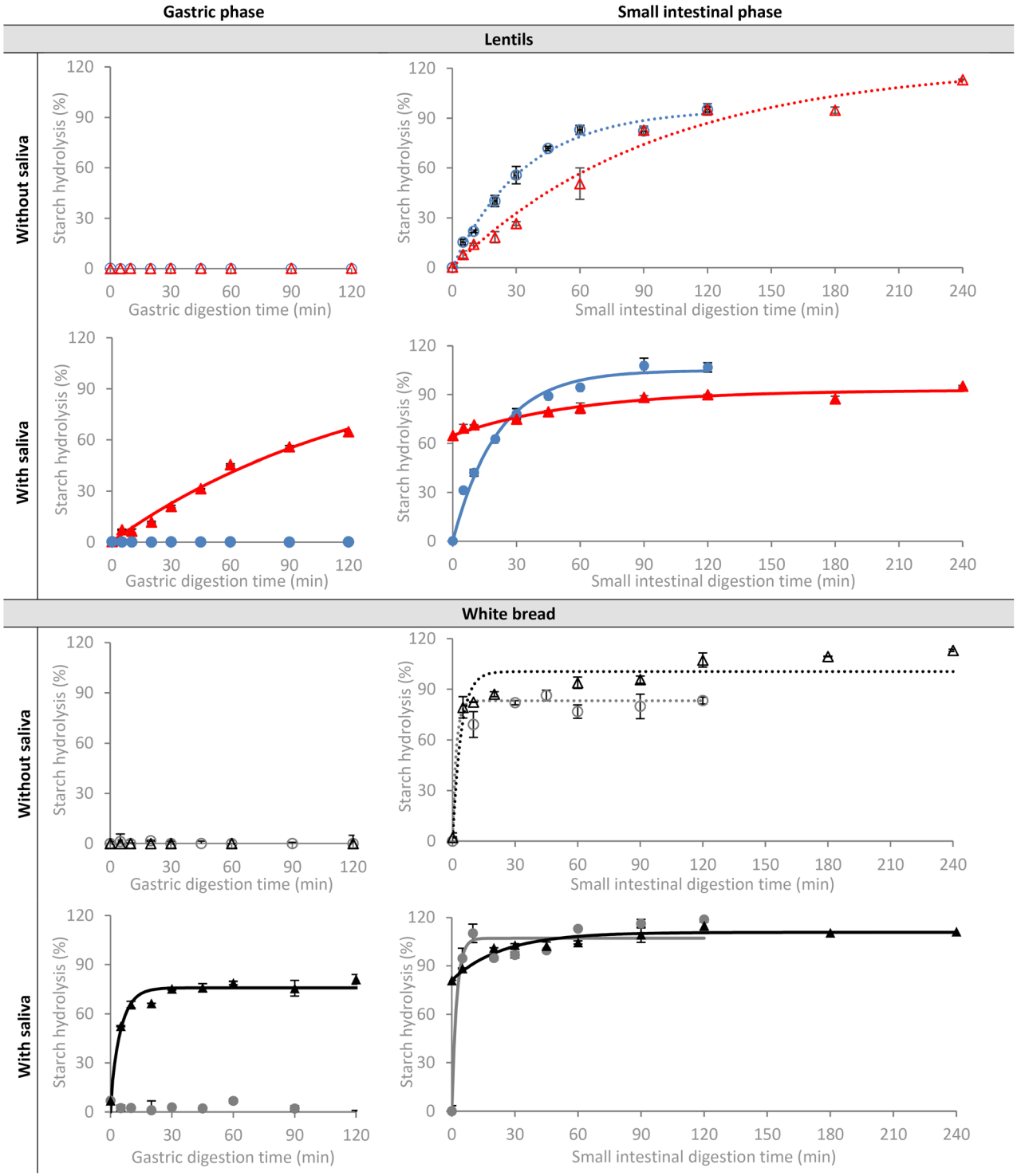

Fig. 3 Starch hydrolysis during gastric and small intestinal *in vitro* digestion of cooked lentils and white bread, applying static *in vitro* models relevant for healthy adults (○/● and ○/●, for lentils and bread, resp.) and older adults (△/▲ and △/▲ for lentils and bread, resp.). Open symbols indicate simulations without addition of saliva in the oral phase, while full symbols represent simulations including saliva. The standard deviation of analytical replicates is indicated by error bars.

amylase activity in the healthy adult model. Moreover, a higher proteolytic activity in both the gastric phase (higher pepsin dosage with higher activity due to lower pH) and the small intestinal phase was expected to lead to a more efficient hydrolysis of the protein matrix entrapping starch granules inside lentil cotyledon cells, in turn facilitating amylolysis.

For simulations of white bread digestion without saliva, very similar amylolysis kinetics were obtained for healthy and

Table 3 Estimated kinetic parameters (±standard error) estimated using the fractional conversion model (eqn (1)) for starch hydrolysis during gastric and small intestinal static *in vitro* digestion applying models relevant for healthy and older adults. The rate constant k and final extent of hydrolysis C_f are reported, with different letters within a column indicating a significant difference between means based on 95% confidence intervals

		Gastric phase			Small intestinal phase		
		k (min^{-1})	C_f (%)	R^2_{adj}	k (min^{-1})	C_f (%)	R^2_{adj}
Lentil							
No saliva	Healthy adult	n.a.	n.a.	n.a.	0.029 ± 0.003^{c}	$95.5 \pm 3.4^{b,c}$	0.99
	Older adult	n.a.	n.a.	n.a.	0.010 ± 0.002^{d}	$122.6 \pm 9.5^{a,b}$	0.99
Saliva	Healthy adult	n.a.	n.a.	n.a.	0.047 ± 0.004^{b}	$104.9 \pm 3.1^{a,b}$	0.99
	Older adult	0.008 ± 0.002^{b}	111.6 ± 23.0^{a}	0.99	$0.017 \pm 0.003^{c,d}$	$92.8 \pm 1.9^{c,d}$	0.99
White bread							
No saliva	Healthy adult	n.a.	n.a.	n.a.	0.118 ± 0.028^{a}	81.7 ± 1.5^{d}	0.99
	Older adult	n.a.	n.a.	n.a.	0.250 ± 0.066^{a}	$100.5 \pm 4.0^{a,b,c}$	0.99
Saliva	Healthy adult	n.a.	n.a.	n.a.	$0.445 \pm 0.168^{a,b}$	$107.1 \pm 3.6^{a,b}$	0.99
	Older adult	0.198 ± 0.027^{a}	75.9 ± 1.7^{a}	0.99	$0.041 \pm 0.008^{b,c}$	110.8 ± 1.5^{a}	0.99

older adult models as well, though amylolysis levels >100% were reached upon longer simulation times (>120 min) for the older adult model. Such high amylolysis levels are not realistic but can be explained by the applied spectrophotometric DNS method. This method quantifies starch digestion products in terms of maltose equivalents. However, this approach can lead to overestimations of the amylolysis extent, especially when higher amounts of glucose are released (compared to *e.g.*, maltose) upon increasing enzymatic incubation times. While this implies that exact digestion extents quantified based on DNS analysis should be interpreted cautiously and different starch metabolites can be better quantified using *e.g.*, chromatographic methods,[61] the DNS method does give valuable insight into amylolysis trends and can allow the comparison of overall digestion trends.[32]

To conclude, the incorporation of saliva and the applied digestion model (healthy *versus* older adult) did significantly affect gastric and subsequent small intestinal kinetics of amylolysis, though similar final (small intestinal) levels were obtained. For both considered food products, gastric digestion at higher pH levels (older adult conditions) lead to a higher degree of amylolysis by the end of gastric digestion. Future research should focus on if and how these changes could also result in a more rapid elevation of blood glucose levels *in vivo* in individuals with a decreased gastric acidification (such as older adults). In this context, rapid starch digestion (*e.g.*, of RDS present in white bread) has been linked to a higher glycaemic response, while slower starch digestion (*e.g.*, of SDS as present in cooked lentils) was linked to a slower and more sustained glucose release *in vivo*.[62] In literature, intact cell walls of pulses have been frequently linked to a slower amylolysis *in vitro*[18] as well as to slower postprandial glucose levels and GI *in vivo* in healthy adults.[23] Even though amylolysis typically occurs faster under older *versus* healthy adult conditions, the current work confirms that cellular encapsulation of starch slows amylolysis under conditions relevant for older adults as well. While *in vivo* validation is necessary before drafting any conclusions regarding physiological response, it can be expected that these (s)lower *in vitro* starch digestion kinetics could potentially cause a (s)lower *in vivo* starch digestion, consequently decreasing the postprandial increase in the blood sugar level.[63] In the context of the older population with a higher prevalence and risk of developing type 2 diabetes, consumption of SDS as present in cooked pulses cooked pulses (rather than, for example, RDS in white bread) could therefore be expected to help maintain normal blood glucose levels.

3.3 Semi-dynamic *in vitro* digestion using digestion models relevant for healthy adults *versus* older adults

In vitro digestion of lentils was repeated applying more advanced semi-dynamic digestion conditions, to obtain further insight into the effect of dynamic gastric conditions (with gradual acidification and pepsin addition) on starch and protein hydrolysis of lentils. Obtained gastric pH profiles are shown in Appendix A, clearly showing a gradual pH decrease to 2 (healthy adult) or 4 (older adult models) by the end of the gastric phase (120 min). The obtained semi-dynamic amylolysis and proteolysis kinetics are discussed in the following paragraphs and compared to those obtained under static simulation conditions.

3.3.1 Protein digestion. Proteolysis during semi-dynamic *in vitro* digestion was quantified in terms of readily bioaccessible small peptides and shown in Fig. 4. Small intestinal digestion data were fitted with the fractional conversion model, with model parameters reported in Table 4.

During the **gastric phase**, proteolysis generally accelerated with proceeding gastric digestion time, due to the gradual decrease in pH and linked increase in pepsin activity in each reactor. Regardless of saliva addition, gastric proteolysis reached higher levels for simulations relevant for healthy compared to older adults, explained by overall lower pH values (Appendix A). Especially from 60 min on, proteolysis increased steeply under healthy adult conditions, due to the decrease of the pH beyond 3.[52] Similar trends could be observed in the presence and absence of saliva. In accordance with this work, a slower gastric proteolysis was reported for tsampa under semi-dynamic older adult compared to healthy adult conditions.[15]

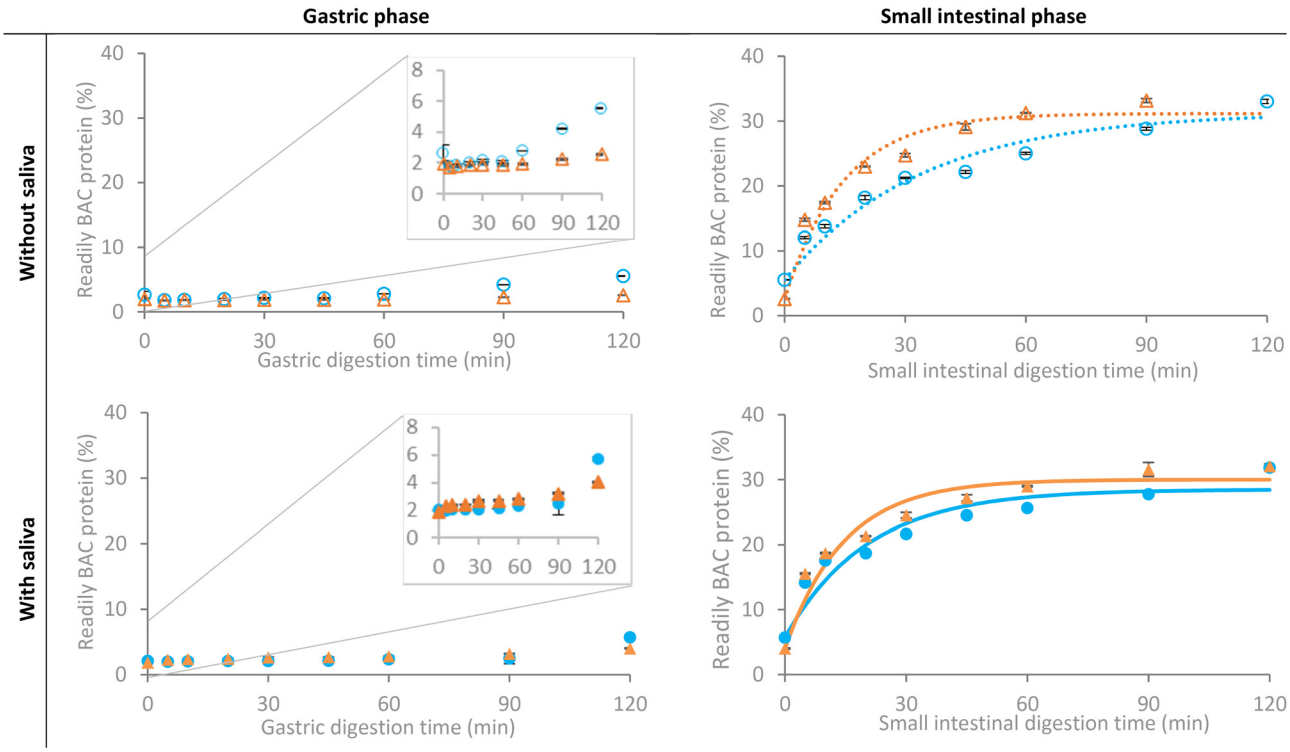

Fig. 4 Protein hydrolysis during gastric and small intestinal *in vitro* digestion of cooked lentils, applying semi-dynamic digestion models relevant for healthy adults (○/●) and older adults (△/▲). Open symbols indicate simulations without addition of saliva in the oral phase, while full symbols represent simulations including saliva. The standard deviation of analytical replicates is indicated by error bars.

Table 4 Estimated kinetic parameters (±standard error) estimated using the fractional conversion model (eqn (1)) for the formation of readily bioaccessible (BAC) protein during small intestinal *in vitro* digestion applying a semi-dynamic model relevant for healthy and older adults. The rate constant k and final extent of hydrolysis C_f are reported, with different letters within a column indicating a significant difference between means based on 95% confidence intervals

		Small intestinal phase		
		k (min^{-1})	C_f (%)	R^2_{adj}
No saliva	Healthy adult	0.030 ± 0.005^a	31.4 ± 2.0^a	0.99
	Older adult	0.069 ± 0.012^a	31.2 ± 1.5^a	0.99
Saliva	Healthy adult	0.049 ± 0.011^a	28.5 ± 1.6^a	0.99
	Older adult	0.069 ± 0.013^a	30.0 ± 1.3^a	0.99

Small intestinal proteolysis followed a similar course for healthy and older adult simulations with and without saliva, and reached similar final levels both, with both C_f and k not significantly different. Based on the insights obtained from Fig. 3, differences in the size distributions of formed small (readily BAC) peptides could be expected to arise during the gastric phase, and gradually disappear again by the end of the small intestinal phase.

3.3.2 Proteolysis kinetics obtained using semi-dynamic *versus* static simulations. The course of proteolysis during semi-dynamic digestion (Fig. 4) was compared to the static simulations (Fig. 1). A comparison between static and semi-dynamic proteolysis kinetics is displayed in Appendix B.

Similar **gastric** levels of proteolysis (5–7%) were reached upon static and semi-dynamic simulations relevant for healthy adults, though gastric proteolysis clearly followed distinct paths. Static healthy adult simulations (with constant pepsin activity and pH of 3) were characterized by an initial increase in degree of proteolysis followed by a long plateau phase (Fig. 1). In contrast, semi-dynamic simulations were characterized by a slow onset (at high pH and low pepsin dosage), after which proteolysis accelerated (Fig. 4). This was especially clear after around 60 minutes of gastric simulation, when pH levels dropped to 3 and below (Appendix A), strongly increasing pepsin activity as explained in the above. This pH decrease below 3 could also affect the susceptibility of lentil protein to hydrolysis through conformational changes.[53]

Similar differences could be observed between static and semi-dynamic simulations relevant for older adults, though a lower extent of proteolysis was measured during the gastric phase as compared to healthy adult models. In general, under static (pH 6) conditions, gastric proteolysis levelled off around 2.5–3% readily BAC protein (Fig. 1). For the semi-dynamic simulation, proteolysis gradually accelerated with the gradual pH decrease (to 4) (Fig. 4), though reaching similar values after 2 h of gastric simulation. As a consequence, it is probable that gradual acidification of gastric contents could accelerate gastric proteolysis *in vivo* as well.

During the subsequent **small intestinal phase**, differences arisen during the gastric phase were compensated, and static and semi-dynamic simulations seemed to generally follow similar courses, reaching similar values after 120 min of simulation. Interestingly, during the first hour of the small intestinal phase of the semi-dynamic older adult simulations, the amount of formed readily BAC peptides seemed higher compared to the static simulations. This is probably caused by the more efficient gastric predigestion caused by the applied pH profile (gradually decreasing to pH 4) in the semi-dynamic case, compared to a high static pH 6 (with very limited pepsin activity) in the static case.[52]

At longer small intestinal digestion times, differences between static and semi-dynamic simulations disappeared, with estimated C_f values not significantly different. Therefore, in accordance to what was already concluded for healthy adult simulations,[12] more simple static simulations can play an important role in initial screening and ranking of samples in terms their overall protein digestibility under conditions relevant for older adults. In contrast, the application of more complex semi-dynamic digestion conditions (such as a gradual pH decrease and pepsin addition) can deliver more in-depth insight into more physiologically relevant proteolysis patterns.

Remarkably, while healthy adult proteolysis curves for static and semi-dynamic simulations without saliva overlapped, higher BAC protein levels were detected for each analysed digestion time for the static simulation upon incorporation of saliva compared to the semi-dynamic one. Earlier (Fig. 1 and Table 2), a higher amount of BAC was also observed in static healthy adult simulations incorporating saliva compared to those without saliva. This was previously explained by possible effects such as the increased accessibility of proteins to proteases upon gradual (orogastric) amylolysis[29,55,56] and the potential precipitation of food proteins in presence of salivary proteins (*e.g.*, mucins) ultimately facilitating proteolysis of more soluble proteins/peptides. Possibly, the activity of salivary amylase as well as the protein precipitation by salivary proteins can be affected by the gastric pH history (static pH 3 *versus* gradual decrease from around 6.5 to 2). In turn, subsequent small intestinal proteolysis could also be differently affected by the gastric pH history. Therefore, additional analyses of the bioaccessible protein fraction (*e.g.*, hydrolysed BAC peptides) could deliver more insight into the absolute amount of protein that is converted into an absorbable form, and the DP of the formed small peptides.

3.3.3 Starch digestion. Amylolysis during semi-dynamic *in vitro* digestion of cooked lentils is shown in Fig. 5. Where relevant, data were modelled using the fractional conversion model, with model parameters reported in Table 5.

In absence of saliva, amylolysis could not take place during the gastric phase. Upon the subsequent addition of pancreatic amylase, a rapid initial amylolysis was observed during the small intestinal phase, levelling off at high extents (88–95%). Small intestinal amylolysis during de older adult simulation

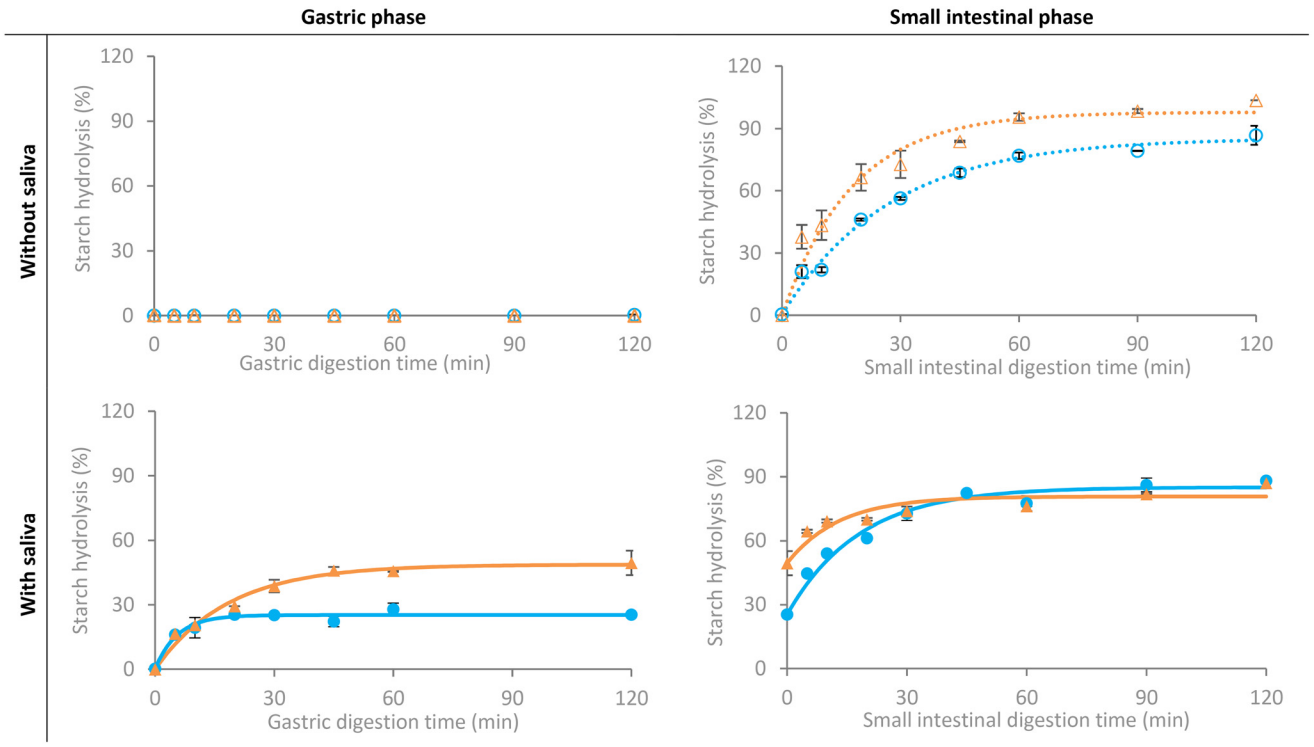

Fig. 5 Starch hydrolysis during gastric and small intestinal *in vitro* digestion of cooked lentils, applying semi-dynamic digestion models relevant for healthy adults (○/●) and older adults (△/▲). Open symbols indicate simulations without addition of saliva in the oral phase, while full symbols represent simulations including saliva. The standard deviation of analytical replicates is indicated by error bars.

Table 5 Estimated kinetic parameters (±standard error) estimated using the fractional conversion model (eqn (1)) for starch hydrolysis during small intestinal *in vitro* digestion applying a semi-dynamic model relevant for healthy and older adults. The rate constant k and final extent of hydrolysis C_f are reported, with different letters within a column indicating a significant difference between means based on 95% confidence intervals

		Gastric phase			Small intestinal phase		
		k (min^{-1})	C_f (%)	R^2_{adj}	k (min^{-1})	C_f (%)	R^2_{adj}
No saliva	Healthy adult	n.a.	n.a.	n.a.	0.035 ± 0.003^a	87.5 ± 3.3^a	0.99
	Older adult	n.a.	n.a.	n.a.	0.062 ± 0.010^a	98.0 ± 4.1^a	0.99
Saliva	Healthy adult	0.118 ± 0.032^a	25.2 ± 0.9^b	0.99	0.056 ± 0.007^a	85.0 ± 2.3^a	0.99
	Older adult	0.054 ± 0.006^b	48.7 ± 1.9^a	0.99	0.077 ± 0.023^a	80.7 ± 2.6^a	0.99

slightly preceded the healthy adult simulation, although this difference was not significant based on both rate constant k and final amylolysis extent C_f. Similar final amylolysis levels were obtained earlier for lentil puree, applying a semi-dynamic digestion protocol considering gradual addition of both gastric and small intestinal enzyme solutions.[41]

Expectedly, starch digestion patterns were significantly affected by saliva incorporation under semi-dynamic conditions, due to gastric pH-profiles allowing and determining salivary amylase activity. For the healthy adult simulation, gastric amylolysis levelled off at ~25% after around 30 minutes. At this time point, the pH inside the reactors dropped below 3.8 (Appendix A), at which salivary amylase activity is significantly decreased.[31,64] Gastric amylolysis under semi-dynamic older adult conditions, reached a plateau ~50%. A significantly higher rate constant k and final extent C_f of gastric amylolysis compared to the healthy adult model could be explained by the slower pH decrease, and thus higher activity of salivary amylase, compared to the healthy adult condition. These differences in amylolysis extent were compensated during the small intestinal phase, reaching high final amylolysis levels under both healthy and older adult semi-dynamic conditions.

3.3.4 Amylolysis kinetics obtained using semi-dynamic *versus* static simulations. The course of amylolysis during semi-dynamic digestion (Fig. 5) can be compared with the static simulations (Fig. 3), as displayed in Appendix C.

For the healthy adult simulations in absence of saliva, logically, no effect of gradual gastric acidification and pepsin addition on starch digestion (Fig. 5) could be observed compared to the static condition (Fig. 3). In contrast, upon saliva incorporation, the consideration of a gradual acidification of the gastric reactor (*i.e.*, semi-dynamic simulation) had a significant effect on gastric amylolysis (reaching ~30% *versus* ~0% for static simulation at gastric pH 3). As explained above, this difference can directly be linked to the pH-dependent activity of salivary amylase.[31,64] These differences were compensated by the end of small intestinal phase. While similar final levels (~100%) were reached, the path of amylolysis (kinetics) was strongly affected by applied (semi-dynamic) digestion conditions.

A comparison between static and semi-dynamic simulations relevant for the older adult population should be made here as well. In absence of saliva, small intestinal amylolysis proceeded faster for the semi-dynamic compared to the static simulation. It is possible a more efficient proteolysis under generally more acidic gastric semi-dynamic conditions[52] could reduce the protein barrier hindering starch-amylase interactions,[30] in turn facilitating amylolysis. In the previous section, a more efficient proteolysis was indeed observed for semi-dynamic *versus* static older adult conditions (Appendix B).

Upon saliva incorporation, a clear difference between the static and semi-dynamic simulations could be observed during the gastric phase once again. In the presence of saliva, a static pH of 6 lead to continuous gastric amylolysis, reaching ~65% after 120 min of the gastric phase. In contrast, gastric amylolysis under semi-dynamic conditions gradually slowed down, levelling off at ~50%, due to the continuous and gradual decrease of salivary amylase activity (with the pH decreasing to 4). While static and semi-dynamic older adult simulation showed clearly different paths of gastrointestinal amylolysis, similar (high) levels of starch hydrolysis were again reached upon 120 min of simulation.

These data confirm that the applied *in vitro* digestion conditions affect the kinetics of starch (and protein) digestion, rather than the final extent of hydrolysis. As noted for the case of (static *versus* semi-dynamic evaluations of) proteolysis, semi-dynamic evaluations can therefore play a pivotal role in better understanding macronutrient digestion kinetics under more realistic conditions. As discussed in section 3.2, these macronutrient hydrolysis patterns can have physiological consequences since higher rates of *in vitro* amylolysis (highly affected by the gastric pH profile) can be hypothesized to lead to faster amylolysis *in vivo*.[63]

Generally, conditions relevant for the altered physiological conditions of older adults gave rise to a more rapid amylolysis compared to healthy adult conditions, while this population group is characterized by a higher prevalence and risk of developing nutrition-related diseases to such as type 2 diabetes. Especially in the context of population groups with delayed gastric acidification, digestion simulations considering salivary amylase combined with more realistic gastric pH profiles can therefore be of importance.

4 Conclusions

The current study aimed to evaluate the effects of gastrointestinal changes occurring upon aging (>65 years of age) on *in vitro*

starch and protein digestion kinetics, by applying static to semi-dynamic *in vitro* simulation approaches. Therefore, firstly, static *in vitro* simulations were employed to evaluate macronutrient digestion in lentils *versus* white bread, considering conditions relevant for healthy adults[33] *versus* digestion conditions adapted for older adults. Secondly, more advanced semi-dynamic simulations adapted to healthy and older adult populations were evaluated, considering gradual gastric acidification and pepsin addition. Thirdly, the relevance and importance of saliva (and salivary amylase) incorporation into static to semi-dynamic digestion simulations relevant for healthy and older adults was evaluated.

As expected, applied *in vitro* conditions significantly affected gastric **amylolysis**. Especially under the (more realistic) simulation conditions considering saliva incorporation and a gradual gastric pH decrease, amylolysis was accelerated under conditions relevant for older adults compared to healthy adults. However, amylolysis profiles obtained under (static) older adult conditions confirmed significantly slowed digestion of lentil compared to white bread starch, plausibly with important consequences for postprandial blood glucose levels. Therefore, future *in vivo* studies should investigate to what level these gastrointestinal changes occurring upon aging are translated into changes in physiological response (*i.e.*, through blood glucose and GI measurements).

Proteolysis patterns were affected by applied static and semi-dynamic digestion conditions as well, mostly due to the pH-dependency of the pepsin activity. Generally, (static and semi-dynamic) conditions relevant for the older population caused a decrease in the extent of gastric proteolysis. Differences in proteolysis patterns induced during the gastric phase were compensated during small intestinal digestion, reaching similar proteolysis endpoints regardless of simulation conditions (both in terms of BAC protein and hydrolysed BAC protein). Though the final extent of proteolysis may be similar regardless of applied digestion model, differences in proteolysis kinetics and peptide release patterns are expected to induce different physiological responses. A follow-up study could aim to quantify time-dependent peptide[37] and amino acid[13,26] release patterns using more in-depth advanced quantification methods.

Importantly, semi-dynamic digestion simulations gave rise to very different macronutrient digestion behaviour as compared to static simulations. On the one hand, final levels of amylolysis and proteolysis were generally similar for static and semi-dynamic simulations. For simulations relevant for both healthy and older adults, simple static simulations are therefore suitable for initial screening and ranking of samples with regards to their macronutrient digestibility. On the other hand, semi-dynamic simulations deliver additional insight into more physiologically relevant digestion processes. In that regard, the complexity of applied digestion simulations should therefore be selected based on the aim of the study and required information level[12,30]

Since the focus of this work was to study the effect of altered gastrointestinal conditions, a standardized bolus was used throughout all simulations. However, development of an oral phase relevant for the oral situation of older adults, as is increasingly being considered in literature,[13,16] can ensure more realistic simulations and resulting digestion kinetics. Importantly, the current work proves the importance of incorporating saliva (or salivary amylase) in such improved oral simulations, in particular when low or gradual gastric acidification is considered in the digestion simulation of starch-rich foods.

Author contributions

D. Duijsens: conceptualization, methodology, formal analysis, investigation, writing – original draft. S. H. E. Verkempinck: writing – review & editing. E. Somers: investigation. M. E. Hendrickx: writing – review & editing. T. Grauwet: conceptualization, supervision, writing – review & editing.

Conflicts of interest

Authors declare no conflicts of interest.

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References

1 OECD. *Population (indicator) [Internet]*, OECD ILibrary, 2023. DOI: **10.1787/d434f82b-en**.
2 I. Dumic, T. Nordin, M. Jecmenica, M. Stojkovic Lalosevic, T. Milosavljevic and T. Milovanovic, Gastrointestinal tract disorders in older age, *Can. J. Gastroenterol. Hepatol.*, 2019, **2019**, 1–19.
3 A. M. Milan and D. Cameron-Smith, Digestion and Postprandial Metabolism in the Elderly [Internet], in *Advances in Food and Nutrition Research*, Elsevier Inc., 1st edn, 2015, vol. 76, pp. 79–124.
4 D. Rémond, D. R. Shahar, D. Gille, P. Pinto, J. Kachal, M. A. Peyron, *et al.*, Understanding the gastrointestinal tract of the elderly to develop dietary solutions that prevent malnutrition, *Oncotarget*, 2015, **6**(16), 13858–13898.
5 C. Shani Levi and U. Lesmes, Bi-compartmental elderly or adult dynamic digestion models applied to interrogate protein digestibility, *Food Funct.*, 2014, **5**(10), 2402–2409.
6 J. E. Morley, J. M. Argiles, W. J. Evans, S. Bhasin, D. Cella, N. E. P. Deutz, *et al.*, Nutritional recommendations for the management of sarcopenia, *J. Am. Med. Dir. Assoc.*, 2010, **11**(6), 391–396.

7 C. Nowson and S. O'Connell, Protein requirements and recommendations for older people: A review, *Nutrients*, 2015, **7**(8), 6874–6899.
8 B. Geloneze, M. D. S. De Oliveira, A. C. J. Vasques, F. S. Novaes, J. C. Pareja and M. A. Tambascia, Impaired incretin secretion and pancreatic dysfunction with older age and diabetes, *Metabolism*, 2014, **63**(7), 922–929.
9 R. Basu, E. Breda, A. L. Oberg, C. C. Powell, C. D. Man, A. Basu, *et al.*, Mechanisms of the age-associated deterioration in glucose tolerance: Contribution of alterations in insulin secretion, action, and clearance, *Diabetes*, 2003, **52**(7), 1738–1748.
10 S. H. Ley, O. Hamdy, V. Mohan and F. B. Hu, Prevention and management of type 2 diabetes: Dietary components and nutritional strategies, *Lancet*, 2014, **383**(9933), 1999–2007.
11 Y. Tamura, T. Omura, K. Toyoshima and A. Araki, Nutrition management in older adults with diabetes: A review on the importance of shifting prevention strategies from metabolic syndrome to frailty, *Nutrients*, 2020, **12**(11), 1–29.
12 D. Duijsens, K. Pälchen, J. Guevara-Zambrano, S. H. Verkempinck, M. Infantes-Garcia, M. Hendrickx, *et al.*, Strategic choices for in vitro food digestion methodologies enabling food digestion design, *Trends Food Sci. Technol.*, 2022, **126**, 61–72.
13 E. Hernández-Olivas, S. Muñoz-Pina, A. Andrés and A. Heredia, Impact of elderly gastrointestinal alterations on in vitro digestion of salmon, sardine, sea bass and hake: Proteolysis, lipolysisand bioaccesibilityt of calcium and vitamins, *Food Chem.*, 2020, **326**, 127024.
14 E. Hernández-Olivas, S. Muñoz-Pina, J. García-Hernández, A. Andrés and A. Heredia, Impact of common gastrointestinal disorders in elderly on in vitro meat protein digestibility and related properties, *Food Biosci.*, 2022, **46**, 12.
15 L. Li, C. Baima, J. Jiang, Z. Liu, J. Wang, X. D. Chen, *et al.*, In vitro gastric digestion and emptying of tsampa under simulated elderly and young adult digestive conditions using a dynamic stomach system, *J. Food Eng.*, 2022, **327**, 111054.
16 O. Menard, U. Lesmes, C. S. Shani-Levi, A. Araiza Calahorra, A. Lavoisier, M. Morzel, *et al.*, Static in vitro digestion model adapted to the general older adult population: an INFOGEST international consensus, *Food Funct.*, 2023, **14**, 4569–4582.
17 C. Shani-Levi, P. Alvito, A. Andrés, R. Assunção, R. Barberá, S. Blanquet-Diot, *et al.*, Extending in vitro digestion models to specific human populations: Perspectives, practical tools and bio-relevant information, *Trends Food Sci. Technol.*, 2017, **60**, 52–63.
18 D. Duijsens, S. Gwala, A. P. Pallares, K. Pälchen, M. Hendrickx and T. Grauwet, How postharvest variables in the pulse value chain affect nutrient digestibility and bioaccessibility, *Compr. Rev. Food Sci. Food Saf.*, 2021, **20**(5), 5067–5096.
19 A. Pallares Pallares, B. Loosveldt, S. N. Karimi, M. Hendrickx and T. Grauwet, Effect of process-induced common bean hardness on structural properties of in vivo generated boluses and consequences for in vitro starch digestion kinetics, *Br. J. Nutr.*, 2019, **122**(4), 388–399.
20 D. Duijsens, K. Pälchen, A. De Coster, S. H. E. Verkempinck, M. E. Hendrickx and T. Grauwet, Effect of manufacturing conditions on in vitro starch and protein digestibility of (cellular) lentil-based ingredients, *Food Res. Int.*, 2022, **158**, 111546.
21 C. H. Edwards, P. Ryden, G. Mandalari, P. J. Butterworth and P. R. Ellis, Structure–function studies of chickpea and durum wheat uncover mechanisms by which cell wall properties influence starch bioaccessibility, *Nat. Food*, 2021, **2**(2), 118–126.
22 T. Berg, J. Singh, A. Hardacre and M. J. Boland, The role of cotyledon cell structure during in vitro digestion of starch in navy beans, *Carbohydr. Polym.*, 2012, **87**(2), 1678–1688.
23 M. Cai, C. H. Edwards, M. Tashkova, S. Tejpal, D. Blunt, I. Garcia-Perez, *et al.*, Cell wall matrices in chickpeas and their effects on starch digestion and postprandial metabolism, *Proc. Nutr. Soc.*, 2023, **82**(OCE1), 2023.
24 K. Pälchen, W. L. P. Bredie, D. Duijsens, A. I. Alfie, M. Hendrickx, A. Van Loey, *et al.*, Effect of processing and microstructural properties of chickpea-flours on in vitro digestion and appetite sensations, *Food Res. Int.*, 2022, **157**, 111245.
25 R. R. R. Bhattarai, S. Dhital, P. Wu, X. D. Chen and M. J. Gidley, Digestion of isolated legume cells in a stomach-duodenum model: Three mechanisms limit starch and protein hydrolysis, *Food Funct.*, 2017, **8**(7), 2573–2582.
26 N. Perez-Moral, S. Saha, A. M. Pinto, B. H. Bajka and H. Edwards, In vitro protein bioaccessibility and human serum amino acid responses to white bread enriched with intact plant cells, *Food Chem.*, 2022, 134538.
27 S. Melchior, M. Moretton, M. Alongi, S. Calligaris, M. C. Nicoli and M. Anese, Comparison of protein in vitro digestibility under adult and elderly conditions: The case study of wheat, pea, rice, and whey proteins, *Food Res. Int.*, 2023, **163**, 112147.
28 S. Ribes, M. Genot, L. Aubry, P. Talens, A. Vénien, V. Santé-Lhoutellier, *et al.*, Oral impairments decrease the nutrient bioaccessibility of bread in the elderly, *Food Hydrocolloids*, 2023, **135**, 11.
29 M. Moretton, M. Alongi, S. Melchior and M. Anese, Adult and elderly in vitro digestibility patterns of proteins and carbohydrates as affected by different commercial bread types, *Food Res. Int.*, 2023, **167**, 112732.
30 K. Pälchen, D. Michels, D. Duijsens, S. T. Gwala, A. K. Pallares Pallares, M. Hendrickx, *et al.*, In vitro protein and starch digestion kinetics of individual chickpea cells: from static to more complex in vitro digestion approaches, *Food Funct.*, 2021, 18–22.
31 D. Freitas, S. Le Feunteun, M. Panouillé and I. Souchon, The important role of salivary α-amylase in the gastric digestion of wheat bread starch, *Food Funct.*, 2018, **9**(1), 200–208.

32 D. Duijsens, S. H. E. Verkempinck, A. De Coster, K. Pälchen, M. Hendrickx and T. Grauwet, How Cooking Time Affects In Vitro Starch and Protein Digestibility of Whole Cooked Lentil Seeds versus Isolated Cotyledon Cells, *Foods*, 2023, **12**(525), 22.
33 A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, *et al.*, INFOGEST static in vitro simulation of gastrointestinal food digestion, *Nat. Protoc.*, 2019, **14**(4), 991–1014.
34 A. I. Mulet-Cabero, L. Egger, R. Portmann, O. Ménard, S. Marze, M. Minekus, *et al.*, A standardised semi-dynamic: in vitro digestion method suitable for food-an international consensus, *Food Funct.*, 2020, **11**(2), 1702–1720.
35 M. d. l. P. Salgado-Cruz, M. Ramírez-Miranda, M. Díaz-Ramírez, L. Alamilla-Beltran and G. Calderón-Domínguez, Microstructural characterisation and glycemic index evaluation of pita bread enriched with chia mucilage, *Food Hydrocolloids*, 2017, **69**, 141–149.
36 S. M. Garvey, J. L. Guice, M. D. Hollins, C. H. Best and K. M. Tinker, Fungal digestive enzymes promote macronutrient hydrolysis in the INFOGEST static in vitro simulation of digestion, *Food Chem.*, 2022, **386**, 132777.
37 D. Duijsens, K. Pälchen, S. Verkempinck, J. Guevara-Zambrano, M. Hendrickx, A. Van Loey, *et al.*, Size exclusion chromatography to evaluate in vitro proteolysis: a case study on the impact of microstructure in pulse powders, *Food Chem.*, 2023, **418**, 135709.
38 A.-I. Mulet-Cabero, L. Egger, R. Portmann, O. Ménard, S. Marze, M. Minekus, *et al.*, A standardised semi-dynamic in vitro digestion method suitable for food – an international consensus, *Food Funct.*, 2020, **11**, 1702–1720.
39 M. Vandenberghe-Descamps, H. Labouré, A. Prot, C. Septier, C. Tournier, G. Feron, *et al.*, Salivary Flow Decreases in Healthy Elderly People Independently of Dental Status and Drug Intake, *J. Texture Stud.*, 2016, **47**(4), 353–360.
40 C. H. Smith, B. Boland, Y. Daureeawoo, E. Donaldson, K. Small and J. Tuomainen, Effect of aging on stimulated salivary flow in adults, *J. Am. Geriatr. Soc.*, 2013, **61**(5), 805–808.
41 S. H. E. Verkempinck, D. Duijsens, D. Michels, J. M. Guevara-Zambrano, M. R. Infantes-Garcia, K. Pälchen, *et al.*, Studying semi-dynamic digestion kinetics of food: Establishing a computer-controlled multireactor approach, *Food Res. Int.*, 2022, **156**, 111301.
42 C. Wang, F. Zhao, Y. Bai, C. Li, X. Xu, K. Kristiansen, *et al.*, Effect of gastrointestinal alterations mimicking elderly conditions on in vitro digestion of meat and soy proteins, *Food Chem.*, 2022, **383**, 132465.
43 T.L Russell, R.R Berardi, J.L Barnett, L.C Dermentzoglou, K.M Jarvenpaa and S.P Schmaltz, *et al.*, *Upper Gastrointestinal pH in Seventy-Nine Healthy, Elderly, North American Men and Women*, Pharmaceutical Research, 1993, vol. 10, pp. 187–196.
44 R. M. Nagler and O. Hershkovich, Relationships between age, drugs, oral sensorial complaints and salivary profile, *Arch. Oral Biol.*, 2005, **50**(1), 7–16.
45 U. M. Nater, C. A. Hoppmann and S. B. Scott, Diurnal profiles of salivary cortisol and alpha-amylase change across the adult lifespan: Evidence from repeated daily life assessments, *Psychoneuroendocrinology*, 2013, **38**(12), 3167–3171.
46 M. Feldman, B. Cryer, K. E. M. C. Arthur, B. A. Huet and E. Lee, Effects of Aging and Gastritis on Gastric Acid and Pepsin, *Gastroenterology*, 1996, **110**, 1043–1052.
47 L. Sams, J. Paume, J. Giallo and F. Carrière, Relevant pH and lipase for in vitro models of gastric digestion, *Food Funct.*, 2016, **7**(1), 30–45.
48 F. Mariotti, D. Tomé and P. P. Mirand, Converting nitrogen into protein - Beyond 6.25 and Jones' factors, *Crit. Rev. Food Sci. Nutr.*, 2008, **48**(2), 177–184.
49 P. M. Nielsen, D. Petersen and C. Dambmann, Improved method for determining food protein degree of hydrolysis, *J. Food Sci.*, 2001, **66**(5), 642–646.
50 M. A. Zahir, V. Fogliano and E. Capuano, Food matrix and processing modulate: In vitro protein digestibility in soybeans, *Food Funct.*, 2018, **9**(12), 6326–6336.
51 S. Gwala, A. Pallares Pallares, K. Pälchen, M. Hendrickx and T. Grauwet, In vitro starch and protein digestion kinetics of cooked Bambara groundnuts depend on processing intensity and hardness sorting, *Food Res. Int.*, 2020, **137**, 109512.
52 D. W. Piper and B. H. Fenton, pH stability and activity curves of pepsin with special reference to their clinical importance, *Gut*, 1965, **6**, 506–508.
53 L. Salelles, J. Floury and S. Le Feunteun, Pepsin activity as a function of pH and digestion time on caseins and egg white proteins under static: In vitro conditions, *Food Funct.*, 2021, **12**(24), 12468–12478.
54 C. Wang, F. Zhao, Y. Bai, C. Li, X. Xu, K. Kristiansen, *et al.*, In vitro digestion mimicking conditions in young and elderly reveals marked differences between profiles and potential bioactivity of peptides from meat and soy proteins, *Food Res. Int.*, 2022, **157**, 111215.
55 Y. Reynaud, C. Buffière, J. David, B. Cohade, M. Vauris, M. Lopez, *et al.*, Temporal changes in postprandial intragastric pH: Comparing measurement methods, food structure effects, and kinetic modelling, *Food Res. Int.*, 2020, **128**, 108784.
56 F. Smith, X. Pan, V. Bellido, G. A. Toole, F. K. Gates, M. S. J. Wickham, *et al.*, Digestibility of gluten proteins is reduced by baking and enhanced by starch digestion, *Mol. Nutr. Food Res.*, 2015, **59**(10), 2034–2043.
57 H. Y. Çelebioğlu, S. Lee and I. S. Chronakis, Interactions of salivary mucins and saliva with food proteins: a review, *Crit. Rev. Food Sci. Nutr.*, 2020, **60**(1), 64–83.
58 L. Chambers, K. McCrickerd and M. R. Yeomans, Optimising foods for satiety, *Trends Food Sci. Technol.*, 2015, **41**(2), 149–160, DOI: **10.1016/j.tifs.2014.10.007**.
59 M. Fried, S. Abramson and J. H. Meyer, Passage of salivary amylase through the stomach in humans, *Dig. Dis. Sci.*, 1987, **32**(10), 1097–1103.
60 D. Freitas and S. Le Feunteun, Oro-gastro-intestinal digestion of starch in white bread, wheat-based and gluten-free pasta: Unveiling the contribution of human salivary α-amylase, *Food Chem.*, 2019, **274**, 566–573.

61 S. Gwala, I. Wainana, A. Pallares Pallares, C. Kyomugasho, M. Hendrickx and T. Grauwet, Texture and interlinked post-process microstructures determine the in vitro starch digestibility of Bambara groundnuts with distinct hard-to-cook levels, *Food Res. Int.*, 2019, **120**, 1–11.
62 E. E. J. G. Aller, I. Abete, A. Astrup, M. J. Alfredo and M. A. van Baak, Starches, sugars and obesity, *Nutrients*, 2011, **3**(3), 341–369.
63 F. J. Warren, B. Zhang, G. Waltzer, M. J. Gidley and S. Dhital, The interplay of α-amylase and amyloglucosidase activities on the digestion of starch in in vitro enzymic systems, *Carbohydr. Polym.*, 2015, **117**, 185–191.
64 A. Mackie, A. I. Mulet-Cabero and A. Torcello-Gomez, Simulating human digestion: Developing our knowledge to create healthier and more sustainable foods, *Food Funct.*, 2020, **11**, 9397–9431.