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ABSTRACT

Ageing leads to changes in the functionality of the digestive tract but the effect of age on digestion and absorption of nutrients remains unclear. The objective of this study was to investigate *in vitro* the digestion of two high-protein dairy products similar to cream cheese (24 % w/w proteins, 20 % w/w lipids) with opposite casein to whey protein ratios, 80:20 (WP-20), and 20:80 (WP-80). The new static digestion model adapted to the general older adult population (≥65 y.) proposed by INFOGEST was used, as well as the standard version of the protocol. Kinetics of proteolysis and lipolysis were compared between both models for each product, in the gastric and intestinal phases of digestion. In both cream cheeses, the degree of protein hydrolysis (DH-P) was significantly lower for older adults than for young adults at the end of the gastric phase (−19 % for WP-20, and −44 % for WP-80), and at the end of the intestinal phase (−16 % for WP-20, and −20 % for WP-80). The degree of lipid hydrolysis (DH-L) was also significantly lower for older adults than for young adults at the end of the digestion for WP-20 (−30 %), but interestingly it was not the case for WP-80 (similar DH-L were measured). Free fatty acids were also released faster from WP-80 than from WP-20 in both digestion conditions: after 5 min of intestinal digestion DH-L was already ≈32 % for WP-80 against 14 % for WP-20. This was attributed to the opposite casein to whey protein ratios, leading to the formation of different gel structures resulting in different patterns of deconstruction in the gastrointestinal tract. This study highlights the fact that it is essential to carefully consider the composition, structure, and digestibility of foods to develop products adapted to the specific needs of the older adult population.

1. Introduction

Malnutrition is a highly prevalent condition among older adults (Dent, Wright, Woo, & Hoogendijk, 2023; Norman, Haß, & Pirlich, 2021), particularly among those who are hospitalized (22 %) or in rehabilitation care units (29 %), and for those who live in nursing homes (18 %) or in long-term care facilities (28.7 %) (Cereda et al., 2016). The cause of malnutrition in older adults is complex and probably multi-factorial; among others, it can be related to physiological changes (e.g., reduced taste or olfactory function, altered hormone signaling, etc.), a reduced access to nutritious food, and comorbidities (Cereda et al., 2016). Ageing leads to changes in the functionality of the digestive tract (Lee et al., 2021; Menard et al., 2023), which may also contribute to the vulnerability of older adults to malnutrition. However, the effect of age on digestion and absorption of nutrients remains unclear (Makran et al., 2022).

Studies investigating the influence of ageing on the digestibility of food are scarce. With regard to dairy products, the few studies that have been carried out have focused on the digestion of proteins (Aalaei, Khakimov, De Gobba, & Ahrné, 2021a, 2021b; Hernández-Olivas, Muñoz-Pina, Sánchez-García, Andrés, & Heredia, 2020; Lavoisier et al., 2023; Melchior et al., 2023). According to these studies, the digestion of milk proteins tends to be reduced in older adults, especially during the gastric phase. However, different *in vitro* parameters were used (pH, enzyme activities, duration, etc.) which limits the comparison of the results obtained. To our knowledge, only one study has investigated milk fat digestion under *in vitro* gastrointestinal conditions relevant to older adults. (Hernández-Olivas, Muñoz-Pina, Sánchez-García, et al., 2020) observed that lipid hydrolysis from fresh and aged cheeses was higher when changes were made to mimic the digestion of older adults. This higher lipolysis in older adults was attributed to the longer duration of the intestinal phase, the decrease in bile salt concentration and

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pancreatin activity (i.e., divided by 2 compared to standard) did not seem to influence lipids hydrolysis.

Recently, a consensus has been reached in the framework of the INFOGEST international network about the physiological parameters that need to be considered to simulate digestive conditions relevant to older adults (≥65 years old) in a static *in vitro* set-up (Menard et al., 2023). The key modifications that were proposed are an increase in the pH and duration of the gastric phase, lower digestive enzyme activities in the gastric and intestinal phases, and a decrease in the concentration of bile salts. This adapted version of the protocol has already been successfully used to study the digestion of protein-rich fermented dairy products (Lavoisier et al., 2023), fermented lentils and quinoa (Sánchez-García et al., 2023) and their flours (Sánchez-García et al., 2024), and cooked lentils (Duijsens, Verkempinck, Somers, Hendrickx, & Grauwet, 2024). We observed that in older adults conditions, a whey protein-based fermented product was less hydrolyzed in the gastric phase than a casein-rich *Skyr* (Lavoisier et al., 2023). As many products developed to prevent malnutrition and promote muscle health in older adults are dairy-based and/or enriched in whey proteins (Devries & Phillips, 2015; Nebbia et al., 2023), the effect of ageing on the digestion of different types of dairy matrices should be better understood.

With this in mind, the objective of this study was to investigate the influence of age on the digestion of two high-protein cream cheeses, with opposite caseins to whey proteins ratios (80:20, which is the ratio found in bovine milk, and 20:80 in order to increase leucine supply to promote muscle health). Products were digested *in vitro* using the standard static INFOGEST model of digestion for adults (Brodkorb et al., 2019), and the recently established version of this model for the general older adult population (Menard et al., 2023). The influence of age on the rate and extent of proteolysis and lipolysis for each product was assessed in the gastric and intestinal phases. To our knowledge, it is the first time that the impact of age on dairy lipids digestion is investigated following the new recommendations of the INFOGEST network. This study should improve our understanding of the digestion of high-protein and high-fat dairy products in the ageing gastrointestinal tract, and provide background information for the development of innovative foods useful for preventing malnutrition in the elderly.

2. Materials & methods

2.1. Materials

Whey proteins (Pronativ® 95) and micellar caseins (micellar casein isolate 88 %) were purchased from Lactalis Ingredients (Bourgbarré, France), the anhydrous milk fat was provided by Eurial Food Service & Industry (Nantes, France), JOHA K emulsifying salts containing diphosphates (E 450) and phosphoric acid (E 338) were from BK Giulini GmbH (Ladenburg, Germany), cheese flavoring was provided by Creanova Flavors (Saint Grégoire, France). All other chemicals and enzymes were purchased from Sigma Aldrich (Saint Quentin Fallavier, France), unless otherwise stated. The Rabbit Gastric Extract (RGE) was provided by Lipolytech (Marseille, France).

2.2. Cream cheese production

Both products contained 24 % (w/w) of milk proteins, 20 % (w/w) of milk fat, 1 % (w/w) cheese flavoring, 0.6 % (w/w) of salt (NaCl), 0.5 % (w/w) of calcium carbonate ($CaCO_3$) or emulsifying salts, and distilled water (dry matter = 48.7 ± 0.7 %). Two cream cheeses were produced with different caseins to whey proteins ratios: WP-80 contained 20 % (w/w) of caseins and 80 % (w/w) of whey proteins, and WP-20 contained 80 % (w/w) of caseins and 20 % (w/w) of whey proteins.

Due to their different content in whey proteins, two different procedures were followed to produce the samples. WP-80 was manufactured by melting first the anhydrous milk fat at 65 °C for 5 min in a food processor (Thermomix®, Vorwerk, Wuppertal, Germany). Then, protein powders, salts and water were added and the mixture was stirred at approx. 300 rpm (corresponding to speed 3 on the food processor) and 50 °C for 75 min. This mix was then heated up to 60 °C for 15 min, and homogenized at approximately 150 bars. Cheese flavoring was added to the homogenized mixture before transferring it to 26 mm diameter synthetic casings (Darmini Darmex Casing Caliber 28, MCM Casing, Lucenay, France). These were then immersed in a water bath at 70 °C for 35 min to form a gel, cooled down to 20 °C in an ice water bath, and stored at 4 °C (final pH = 6.8). WP-20 was produced by directly mixing all the ingredients in the food processor at 50 °C. The mixture was first stirred at approx. 300 rpm and 50 °C for 75 min and then heated up to 70 °C for 30 s to form a gel before its transfer into plastic containers. Samples were finally cooled down to 20 °C in an ice water bath, and stored at 4 °C (final pH = 6.1). One bulk batch of each cream cheese type was produced, divided in several containers and used for *in vitro* digestion experiments in less than 30 days (this shelf life has been evaluated from a microbiological point of view).

2.3. Static in vitro digestion

Two static *in vitro* digestion models were used to study the impact of age on samples hydrolysis in the gastrointestinal tract. Digestion by adults < 65 years old (called "young adults") was simulated according to (Brodkorb et al., 2019), and digestion by adults ≥ 65 years old (referred to as "older adults") was carried out according to (Menard et al., 2023). Products were semi-solid soft gels, and therefore we did not simulate chewing. Neither α-amylase nor mucins were included in the oral phase. The oral fluid to food ratio was 1:1 (weight of SSF:dry weight of food), considering that 10 g of cream cheese sample was used in each *in vitro* digestion experiment, and the mean dry weight of samples was 50.8 % for WP-80 and 51.8 % for WP-20.

Digesta samples were collected before digestion (G0), after 30, 60, 120 and 180 min (the latter for older adults only) in the gastric phase (G30, G60, G120 and G180, 2 mL withdrawn at each timepoint), and after 5, 15, 30, 60 and 120 min in the intestinal phase (I5, I15, I30, I60 and I120, 2 mL withdrawn at each timepoint). Pepsin activity was inhibited by Pepstatin A (50 μL of a 0.72 mM solution in methanol added /mL of digesta), while trypsin and chymotrypsin activity were inhibited by Pefabloc (50 μL of a 0.1 M solution in water added /mL of digesta). 4-Bromophenylboronic acid (1 M in methanol) was used to inhibit lipase activity in the gastric phase (50 μL /mL of digesta) and in the intestinal phase (5 μL /mL of digesta). Three replicates were carried out for each experimental condition. Digesta samples were stored at −20 °C until further analysis.

2.4. Biochemical analysis of digesta samples

2.4.1. Degree of hydrolysis of proteins (OPA method)

The degree of hydrolysis of proteins (DH-P) was evaluated with the o-phthalaldehyde (OPA) method adapted from (Church, Swaisgood, Porter, & Catignani, 1983) as previously described (Lorieau et al., 2018). The digesta sample was centrifuged (10,000 g for 20 min at 4 °C) and the aqueous part of the supernatant was recovered for analysis. A Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the absorbance at 340 nm after 10 min of contact between 100 μL of the OPA reagent (0.5 % w/v SDS, 0.25 mg/mL OPA, 7 mM DTT, 50 mM sodium tetraborate) and 50 μL of the diluted samples, at 37 °C in a 96-well plate. A methionine standard solution (0 to 2 mM) was used as calibration curve. The measurements were carried out in duplicate on each triplicate from the digestion experiments. The DH-P was calculated as follows:

$$DH-P(\%) = \frac{\left[NH_{2(t)}\right] - \left[NH_{2(t0)}\right] - \left[NH_{2(fluids)}\right]}{\left[NH_{2(total)}\right] - \left[NH_{2(t0)}\right]} \times 100$$

Where $[NH_{2\ (t)}]$ was the concentration in primary amines quantified at

each digestion time (mg NH_2/ L digesta), [$NH_{2\ (t0)}$] the concentration in primary amines present in the product before digestion (mg NH_2/ L digesta), [$NH_{2\ (fluids)}$] the concentration in primary amines in the digestive fluids (including enzymes: pepsin and RGE in the gastric phase; pepsin, RGE, and pancreatin in the intestinal phase), and [$NH_{2\ (total)}$] the concentration in primary amines in the undigested product after total acid hydrolysis in 6 N HCl at 110 °C for 24 h.

2.4.2. Protein hydrolysis (SDS-PAGE)

The protein composition of the digesta samples was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described by (Lavoisier et al., 2023). SDS-PAGE analysis was performed on the thawed digesta samples, without centrifugation, using 8–16 % Stain-Free Mini-PROTEAN TGX 12-well precast polyacrylamide gels (Bio-Rad Laboratories Inc., Hercules, CA). Samples were diluted in a (2x) Laemmli sample buffer (Bio-Rad Laboratories Inc.) under reducing conditions (with a 0.5 M DTT solution and 10 min at 95 °C). Five mg of total protein (excluding enzymes) were then loaded into each well of the gel. Unstained Precision Plus Protein Standards (Bio-Rad Laboratories Inc. Hercules, CA) were used for molecular weight calibration. Electrophoresis was performed first at 100 V for 15 min, and then at 200 V for 30 min. After 5 min of photoactivation, bands were visualized by UV excitation and scanned with a ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc., Hercules, CA). The measurements were carried out in triplicate. Image Lab software (v6.1) was used for image visualization and processing, and no saturation was observed on the images. Protein bands were quantified by extracting the band volume, and at each time point, calculating the percentage relatively to the initial volume at G0.The main protein bands were identified by comparing lane profiles with literature data (Jovanovic, Barac, Macej, Vucic, & Lacnjevac, 2007; Sharma et al., 2021).

2.4.3. Amino acids quantification

Samples collected at I5, I30, I60 and I120 were centrifuged at 10,000 g for 20 min at 4 °C. Total and free amino acids (AA) in the aqueous portion of the supernatant were determined by cation exchange chromatography on a Biochrom 30 automatic AA analyzer (Biochrom Ltd., Cambridge, UK) as previously described by (Lorieau et al., 2018). The bioaccessibility of each essential AA was calculated as follows:

$$\text{AA bioaccessibility}\ (\%) = \frac{[FAA_{(t)}]}{[TAA_{(t)}]} \times 100$$

where [FAA(t)] is the concentration in free AA at each digestion time (mg/L of intestinal digesta), [TAA(t)] the concentration of AA at each digestion time after total acid hydrolysis in 6 N HCl at 110 °C for 24 h (mg/L of intestinal digesta). Total and free AA in the digestive fluids (including enzymes and inhibitors) were determined by the same method and subtracted. The measurements were carried out in duplicate on each triplicate from the digestion experiments. Tryptophan was not quantified.

2.4.4. Lipids extraction and samples preparation for GC–MS

The Folch extraction procedure (Folch, Lees, & Stanley, 1957) was used to isolate lipids from the digesta samples (G120 or G180, I5, I15, I30, and I120) and the undigested cream cheese (G0). Briefly, the sample and the internal standards solution (containing TAG-C17, TAG-C11 and TAG-C5 for free fatty acids analysis, or TAG-C13 for total fatty acids analysis) were dispersed into a chloroform–methanol mixture. After stirring for 30 min, extracts were washed first with KCl (0.8 %) and then with a chloroform–methanol-KCl mixture. The solvent phase was finally filtered to obtain the total lipids extract.

Free fatty acids (FFA) were isolated from the Folch extract using a solid-phase extraction column (NH2, 3 mL/500 mg, Macherey-Nagel, Fisher Scientific SAS, Illkirch, France). Neutral lipids were first eluted with an hexan-isopropanol mixture, and FFA were then collected with a diethyl ester-formic acid mixture. Total fatty acids (TFA) were saponified from the Folch extract with sodium methylate at 70 °C for 20 min. Both FFA and TFA were methylated with boron trifluoride at 70 °C for 15 min. The resulting fatty acid methyl esters (FAMEs) were extracted with hexane, and stored at −20 °C until further analysis. Three replicates were carried out for each experimental condition.

2.4.5. Lipids identification and quantification by GC–MS

FFA and TFA were identified and quantified using a Shimadzu gas chromatograph mass spectrometer QP2010 SE (Shimadzu Corp., Kyoto, Japan). equipped with a BPX70 capillary column (120 m, 0.25 mm i.d., 0.25 μm film, Trajan Scientific and Medical, Morrisville, NC, USA). The carrier gas was helium set at a constant flow rate of 2.3 mL min^{-1}. The injection volume was 0.5 μL for TFA measurements and 1 μL for FFA, introduced via a split/splitless injector at 250 ∘C. The oven temperature was programmed to follow a ramped profile from 50 °C to a final temperature of 240 °C, with one plateau at 175 °C. Detection was performed with a mass spectrometer set on SCAN mode, and with ionization by electronic impact. The ion source temperature was set at 200 °C and the interface temperature at 250 °C. The measurements were carried out in duplicate on each triplicate from the digestion experiments. Fatty acids identification was cross-checked by mass spectrometry using the NIST mass spectral database library (2023; www.nist.gov). GCMS solution software (Shimadzu Corp., Kyoto, Japan) was used for data acquisition. The qualitative and quantitative composition of the molecules found was determined using the internal standards added to the samples.

The degree of hydrolysis of lipids (DH-L) was calculated as follows:

$$DH-L(\%) = \frac{[FFAs_{(t)}]}{[TFAs_{(0)}]} \times 100$$

Where [FFA $_{(t)}$] was the FFA detected at each digestion time (μmol/L), and [TFA $_{(0)}$] was the TFA detected in the undigested samples (μmol/L).

2.5. Confocal laser scanning microscopy (CLSM)

The microstructure of both samples was observed at G0 in both digestion models (i.e., at pH 3 or pH 3.7). A Zeiss LSM880 confocal microscope was used, with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective (Carl Zeiss Microscopy, LLC, White Plains, NY, USA). Samples were stained with Nile Red (0.1 % w/v, λex 488 nm, λem 550–590 nm) and Fast Green (1 % w/v, λex 633 nm, λem 635–735 nm) for 10 min at 20 °C. Two single images were obtained, one showing the lipids stained with Nile Red and the other showing the proteins stained with Fast Green. Overlay images are presented in the following section (cf. Fig. 8) but both images were processed and analyzed individually with the open source ImageJ software (Schneider, Rasband, & Eliceiri, 2012). Lipid droplets were segmented from the background using a binary mask, threshold segmentation and morphological image operations (i.e., noise removal and suppression of objects at image boundaries). The *Analyze particles* tool was used to measure particle size (more than 500 particles were considered on each of the three images selected for each condition). For lipid droplets the area of each particle and the median value obtained on each image were used to calculate the estimated *d*50 (in μm, considering spherical particles). With respect to protein particles, the mean Feret diameter (in μm) was considered to evaluate particle size for each experimental condition.

2.6. Statistical analysis

Unless overwise stated, results are shown in terms of the mean ± standard deviation. Statistical significance of the results ($p \leq 0.05$) was tested using Kruskal-Wallis test, and Tukey's multiple pairwise comparison between groups. Data analyses were performed using the R software, version 4.2.2.

3. Results

3.1. Protein digestion

The degree of protein hydrolysis (DH-P) in WP-80 and in WP-20 during the gastric and intestinal phases in both digestion models is presented in Fig. 1. In WP-80, DH-P was significantly lower in older adults than in young adults from G60 and until the end of the digestion (I120). The difference in DH-P between models was more important in the gastric phase than in the intestinal phase: at the end of the gastric phase (G120 vs G180 min) DH-P was reduced by −44 % in older adults, compared to −20 % at I120. DH-P values observed at I120 were relatively low (<35 %), but it seems that the protein hydrolysis had not reached a plateau, as DH-P values were still increasing significantly between I60 and I120 in both models. In WP-20, DH-P was significantly lower in older adults than in young adults throughout digestion. These lower values in DH-P for older adults were similar at the end of the gastric and intestinal phases (−19 %, and −16 %, respectively). DH-P values observed at I120 were also relatively low (<35 %).

The protein profiles of WP-80 and WP-20 before and during digestion in both conditions are presented in Fig. 2. As expected, both products contained mainly caseins, β-lactoglobulin (β-lg), and α-lactalbumin (α-la), in different proportions according to their formulation: bands identified as β-lg (at 16.6 kDa) and α-la (at 12.4 kDa) were more pronounced in WP-80, while bands identified as caseins αs1, and β (at 30.2 and 28.5 kDa, respectively) were more marked in WP-20.

Hydrolysis by pepsin, during the gastric phase, of the main proteins in both cream cheese samples is reported in Fig. 3. Overall, age did not influence significantly β-lg hydrolysis in both samples (Fig. 3a). The intensity of the band corresponding to β-lg remained practically unchanged from G30 until the end of the gastric phase in both samples and in both digestion conditions. However, β-lg was more resistant to pepsin hydrolysis in WP-80 than in WP-20 in the first 30 min of digestion. The decrease in intensity of the band corresponding to α-la was more pronounced (Fig. 3b). However, in both samples and in both digestion conditions, α-la bands were still visible at the end of the gastric phase, meaning that this protein also partially resisted pepsin hydrolysis. In WP-80, α-la hydrolysis was slower in older than in young adults, but not in WP-20.

Overall, caseins were extensively degraded during the gastric phase in both products and in both age models, with a few differences (Fig. 3c and 3d). Bands corresponding to α-s1 and β-casein disappeared rapidly in WP-80. A difference in kinetics was observed between age conditions: casein bands disappeared completely at G60 for older adults compared to G30 for young adults. In WP-20, probably because of their initial higher abundance, the intensity of α-s1 and β-casein bands decreased more progressively. Again, a significant difference was observed between age conditions. Casein bands had completely disappeared at the end of the gastric phase for young adults (G120) but not for older adults (G180).

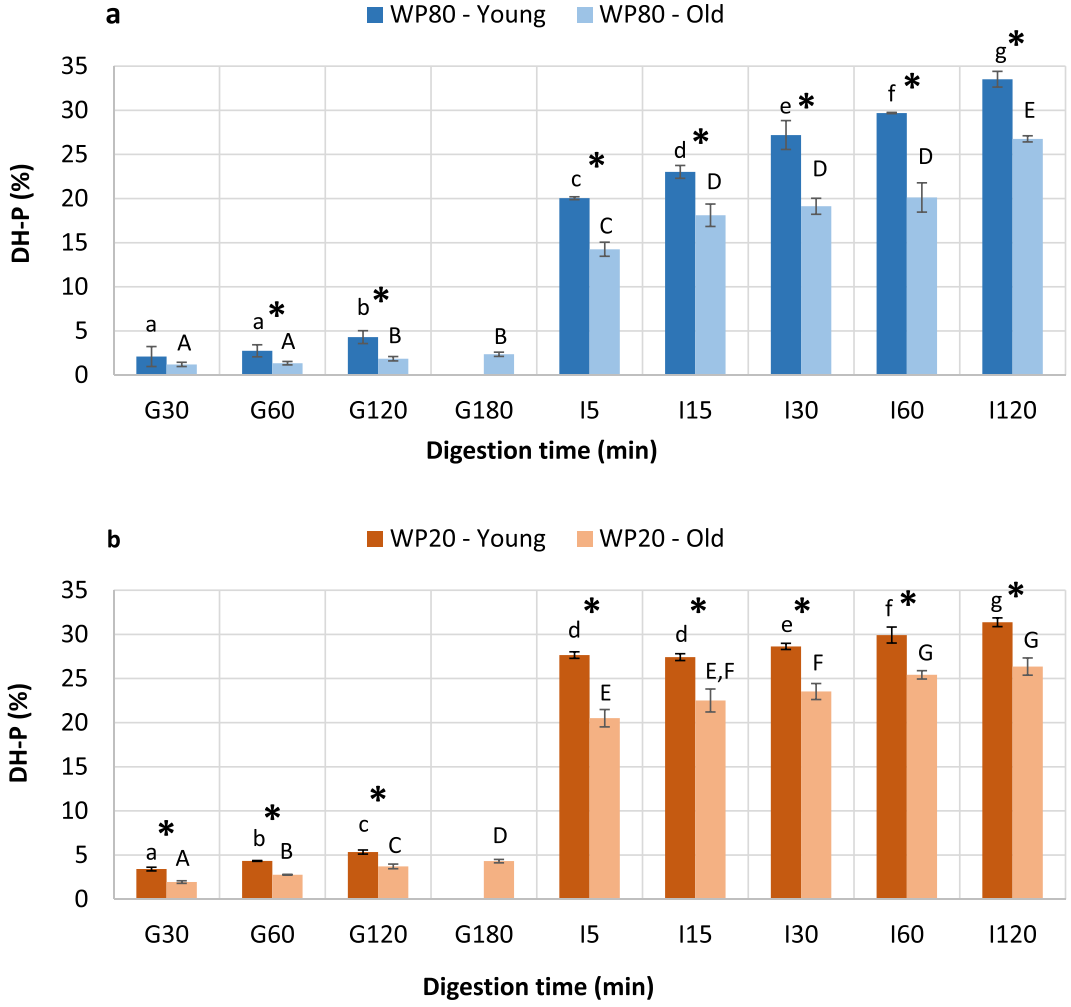

Fig. 1. Degree of protein hydrolysis (DH-P) during the gastric and intestinal phases of digestion, in young and older adult conditions, in (a) the whey protein-rich product (WP-80), and in (b) the casein-rich product (WP-20). Significant differences ($p \leq 0.05$) according to age are marked with a star (*), and according to the digestion time are marked with different letters (lower case for young adult conditions, and upper case for older adult conditions).

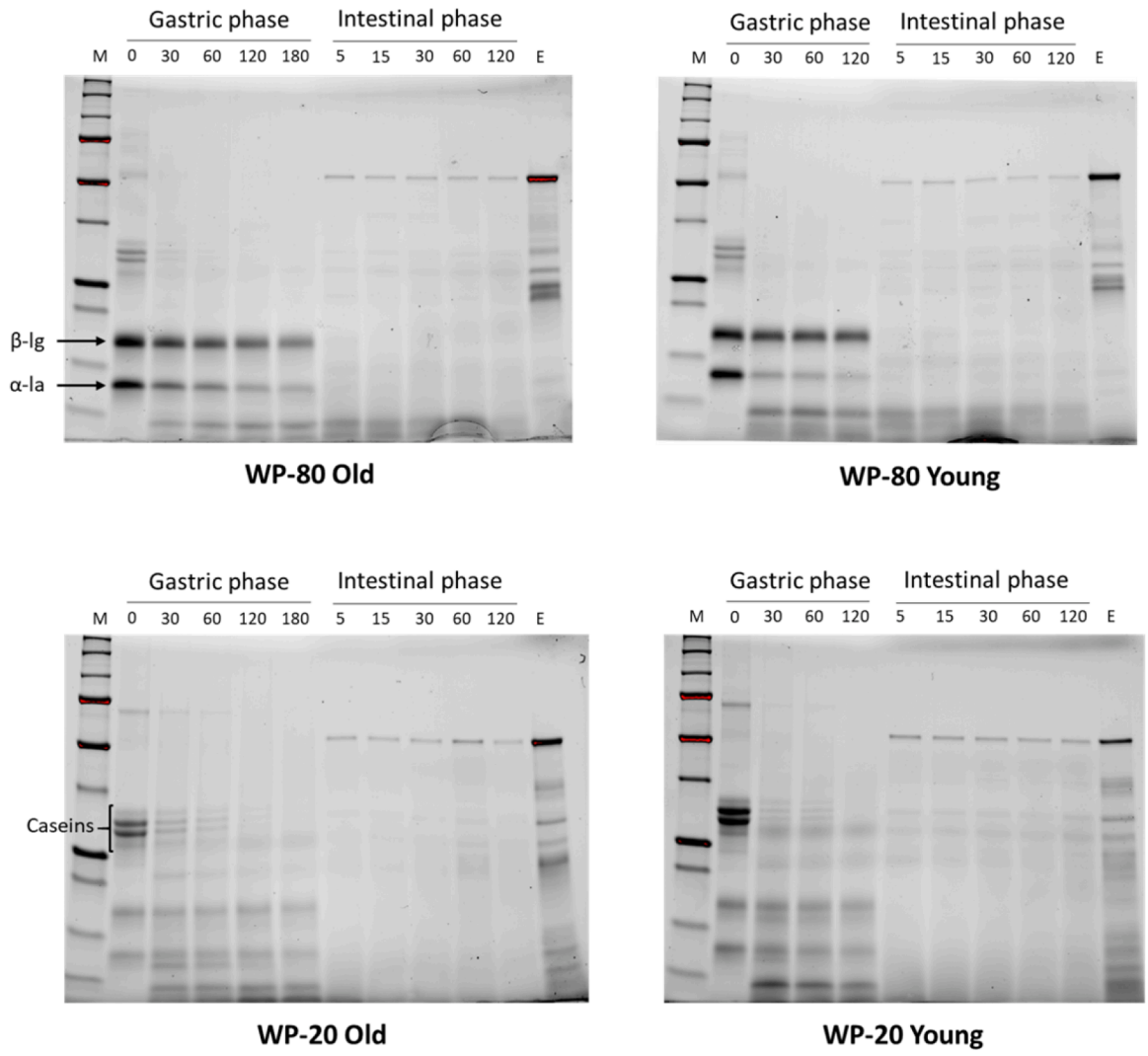

Fig. 2. SDS-PAGE patterns of WP-80 and WP-20 before (G0) and during the gastric and intestinal phases of digestion, in young and older adult conditions. Images are representative of the three repetitions measured for each condition. M = molecular weight standards, E = enzymatic blank sample, β-lg = beta-lactoglobulin, and α-la = alpha-lactalbumin.

In WP-20, containing mainly caseins, new bands appeared at approx. 20 kDa and 15 kDa (Fig. 2). These bands were visible from G30 until the end of the gastric phase, and no significant differences in intensity were measured between older and young adult's conditions. In both samples and in both digestion conditions, new bands < 10 kDa appeared rapidly from G30. They were less abundant overall in older adult conditions. In WP-80, these bands attributed to proteolytic products were still visible in the intestinal phase in both digestion models (Fig. 2).

The composition in essential amino acids of WP-80 and WP-20 before digestion is presented in Table 1, and the bioaccessibility of these essential amino acids at the end of the intestinal phase of digestion are shown in Fig. 4. As expected, WP-80 contained more leucine (Leu, +36 %), lysine (Lys, +31 %), isoleucine (Ile, +11 %), and threonine (Thr, +11 %) than WP-20, but it also contained less histidine (His, −9%), phenylalanine (Phe, −9%), and valine (Val, −4%). No difference in the methionine (Met) content was observed between the two products, and it should be noted that the determination of tryptophan could not be performed with the method used in this study due to degradation during acid hydrolysis.

In WP-80, slightly less Phe and Thr were released in older adults than in young adults at I120, while no significant differences were observed for His, Ile, Leu, Lys, and Val. Met was the only EAA that was slightly more accessible in older adults than in young adults at the end of the digestion. In WP-20 slightly less His, and Thr were released for older adults at I120, while no significant differences were observed in the bioaccessibility of Ile, Leu, Lys, Met, Phe, and Val.

The bioaccessibility of Leu in WP-80 and WP-20 during the whole intestinal phase of digestion are represented in Fig. 5 and Figure S1. In WP-80, Leu bioaccessibility increased continuously during the 120 min of the intestinal digestion in both models, without reaching a plateau. The amount of accessible Leu was slightly lower in older adults than in young adults up to I60, but no significant differences were measured at I120. In WP-20, Leu bioaccessibility did not increase significantly after I30, and no differences in the rate of Leu liberation were observed between age conditions.

3.2. *Lipid digestion*

The degree of lipid hydrolysis (DH-L) in WP-80 and in WP-20 during the gastric and intestinal phases in both digestion models is presented in Fig. 6. In WP-80, lipid hydrolysis started in the gastric phase and almost reached its maximum after only 5 min of intestinal digestion (approximately 30 % at I5, and 35 % at I120, non-significant difference for young adults). Overall, no differences were observed between older and young adults. In WP-20, lipid hydrolysis was very limited in the gastric phase (approx. 1 %) and progressed constantly during the intestinal phase

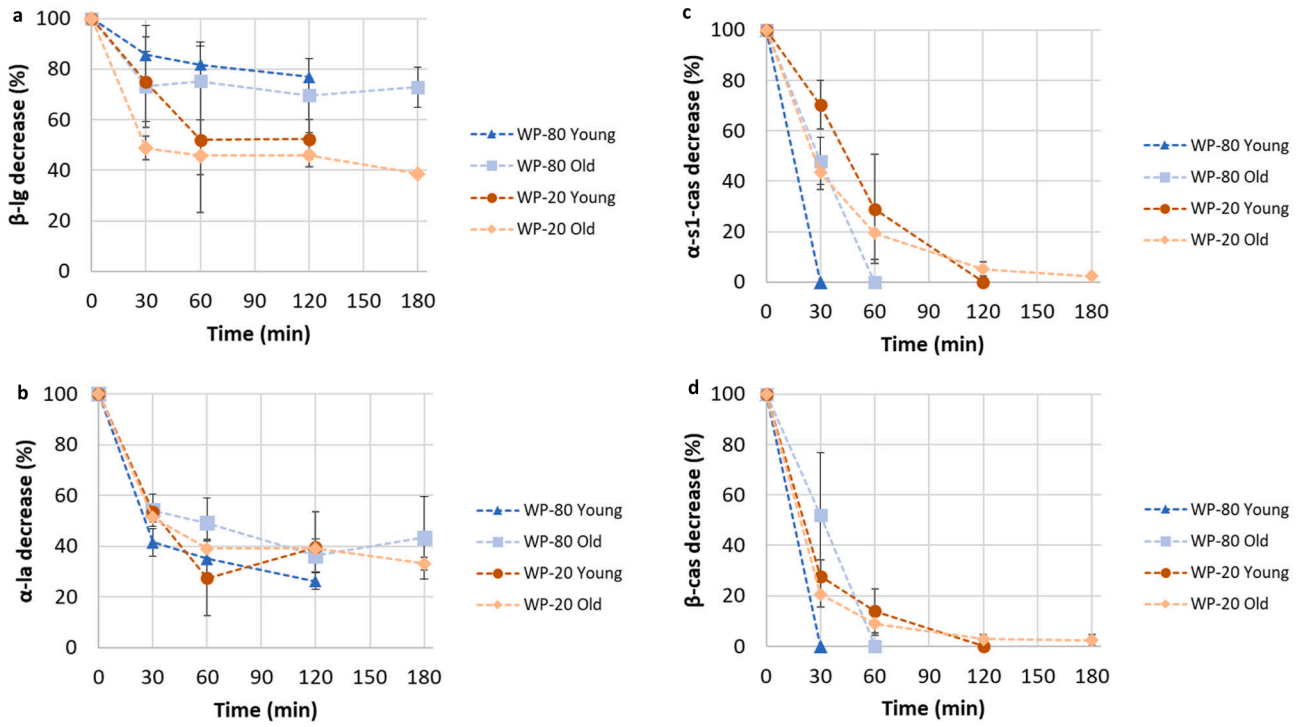

Fig. 3. Protein digestion in the gastric phase, in WP-80 and WP-20 and in young and older adult conditions, reflected by the evolution in time of the band volume relatively to the volume at G0 (%). Bands were attributed to most abundant whey proteins: (a) β-lg = beta-lactoglobulin (mw ≈ 16.5 kDa), and (b) α-la = alpha-lactalbumin (mw ≈ 12.5 kDa), and caseins: (c) α-s1-cas = alpha-s1-casein (mw ≈ 31 kDa), and (d) β-cas = beta-casein (mw ≈ 29 kDa).

Table 1
Composition in essential amino acids of the whey protein-rich cream cheese (WP-80) and of the casein-rich cream cheese (WP-20) before digestion. Results are expressed in g of amino acid /kg of product. Significant differences are marked with a star (*).

Essential amino acids, mean ± std. dev. (g/kg)	WP-80	WP-20
Histidine	5.16 ± 0.06	5.69 ± 0.27
Isoleucine	13.22 ± 0.19	11.94 ± 0.32 *
Leucine	31.28 ± 0.76	23.02 ± 0.85 *
Lysine	25.51 ± 0.45	19.45 ± 0.76 *
Methionine	6.14 ± 0.04	6.02 ± 0.26
Phenylalanine	9.38 ± 0.03	10.26 ± 0.33 *
Threonine	11.53 ± 0.16	10.38 ± 0.34 *
Valine	13.52 ± 0.13	14.02 ± 0.39

without reaching a plateau. In this case, the digestion conditions used had a significant effect on the DH-L: from I30 up to the end of the intestinal phase the DH-L was significantly, and increasingly, lower in older adults than for in young adults. At I120, the DH-L was 38 % lower in the older adults.

Both samples were formulated with the same milk fat and therefore had the same fatty acid (FA) profile before digestion (Figure S2). The main fatty acids found in the samples were the following: palmitic acid (C16:0, 41 %), oleic acid (C18:1n9c, 20 %), myristic acid (C14:0, 14 %), stearic acid (C18:0, 10 %), lauric acid (C12:0, 4 %), and decanoic acid (C10:0, 2 %). Samples contained mainly saturated FA (73 %), and mono-unsaturated FA (23 %). The ω6/ω3 polyunsaturated fatty acid ratio of these samples was 4.59 ± 0.26.

Overall, the FFA released during the gastric phase were mainly saturated (85 to 95 % of the total FFA measured) and mono-unsaturated (5 to 15 % of the total FFA measured). The release of a significant amount of poly-unsaturated FA started in the intestinal phase (<1% before I5, data not shown). The proportions of saturated, mono-, and poly-unsaturated FA released at I120 from each sample and for each digestion model are presented in Fig. 7. In WP-80, the percentage of mono and poly-unsaturated FA released tended to be slightly lower in older adults than in young adults during the whole intestinal phase (data not shown), but no significant differences were observed between models at I120 (Fig. 7a). Similar results were observed in WP-20 (data not shown), but in this case the percentage of mono and poly-unsaturated FA released at I120 was significantly lower in older adults (−4% and −14 %, respectively), and consequently the percentage of saturated FA released was higher (+2%, Fig. 7b).

3.3. Microstructure of the samples at G0

Confocal microscopy images of WP-80 and WP-20 diluted in digestive fluids at pH 3.7 (older adult conditions in the gastric phase at G0) and at pH 3 (young adult conditions in the gastric phase at G0) are presented in Fig. 8. On these images, lipid droplets appear in red and protein aggregates in green. In all samples, lipid droplets were observed in the protein matrix as well as free lipid droplets separated from the protein matrix. Lipid droplets in WP-80 were significantly smaller than in WP-20 at pH 3.7 ($d50$ = 0.52 ± 0.01 μm for WP-80 versus 0.78 ± 0.04 μm for WP-20), and at pH 3 ($d50$ = 0.58 ± 0.02 μm for WP-80 versus 0.69 ± 0.09 μm for WP-20). The effect of pH on lipid droplet size depended on the product: in WP-80 droplets size decreased slightly with increasing pH, while in WP-20 droplet size tended to increase. In fact, some droplet coalescence was visible on WP-20 images at pH 3.7 (Fig. 8c). Regarding protein aggregates, larger particles were measured at pH 3.7 than at pH 3, in both products (mean Feret diam. = 3.1 ± 0.1 μm for WP-80 and 3.6 ± 0.2 μm for WP-20 at pH 3.7, versus 2.6 ± 0.2 μm for WP-80 and 2.9 ± 0.4 μm for WP-20 at pH 3). It should also be noted that in the experiments with WP-20, when pH was lowered from 7 to 3 or 3.7 between the oral and the gastric phase of digestion, the formation of a weak coagulum was observed, which was not the case

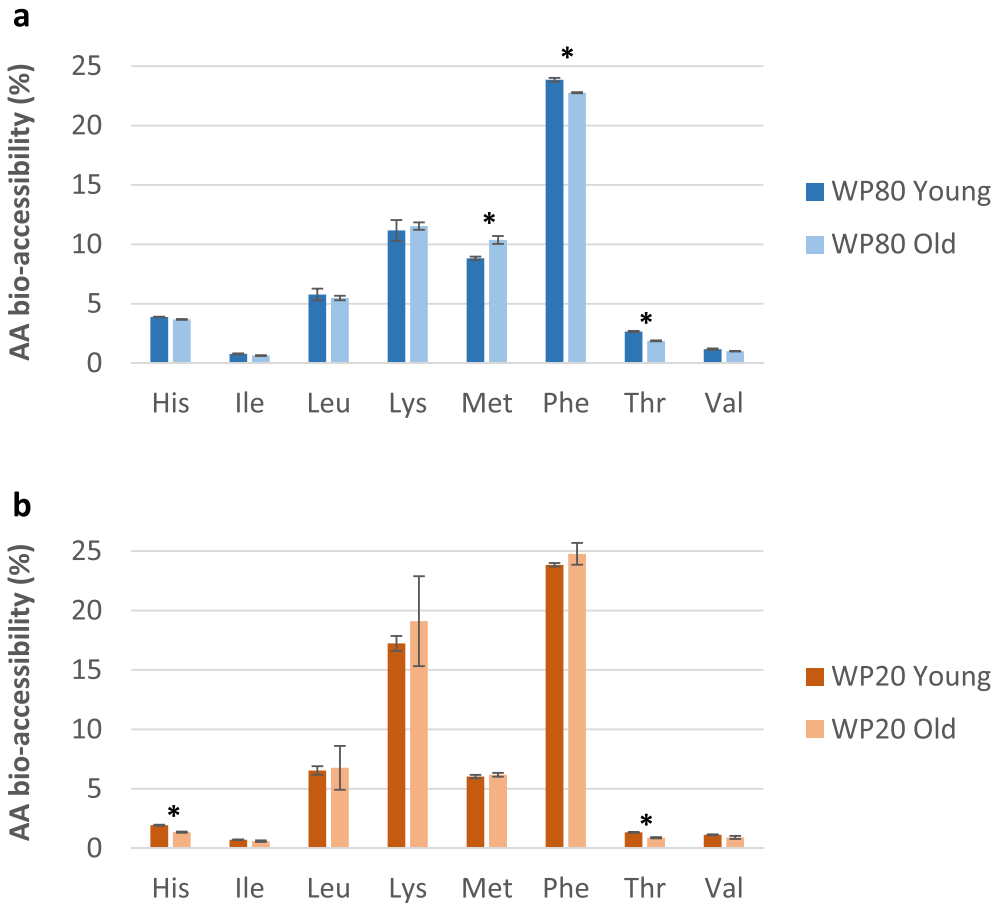

Fig. 4. Bioaccessibility of essential amino acids at the end of digestion (I120) in young and older adult conditions, in (a) WP-80 and in (b) WP-20. His = histidine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Thr = threonine, Val = valine. Significant differences ($p \leq 0.05$) according to age are marked with a star (*).

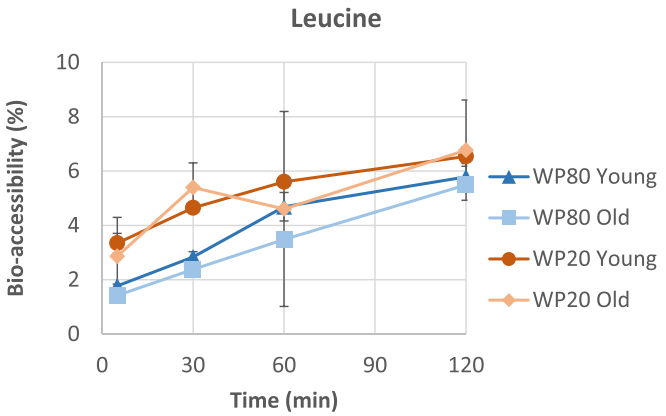

Fig. 5. Leucine liberated during the intestinal phase of digestion in WP-80 and WP-20 and in young and older adult conditions.

with WP-80 (images not shown).

4. Discussion

The main objective of this study is to improve our understanding of proteins and lipids digestion from dairy products in the ageing gastrointestinal tract (GIT). Therefore, the influence of age on the digestion of high-protein cream cheese was studied with two static *in vitro* digestion models representing the conditions in the GIT of young adults (<65 y.) (Brodkorb et al., 2019) or healthy older adults (≥65 y.) (Menard et al., 2023). Based on the assumption that whey proteins may present some nutritional benefits for older adults (Dangin et al., 2003), the high-protein cream cheese samples were formulated with opposite casein to whey protein ratios (80:20 and 20:80).

4.1. Protein digestion

Protein hydrolysis in the gastric phase was reduced in older adults, due to the lower pepsin concentration (i.e., −40 %), and the higher pH (i.e., from 3 to 3.7) used in the adapted protocol compared to the standard. The impact of the reduction in pepsin activity on protein digestion in the gastric phase was higher in the WP-rich product than in the casein-rich product (−44 % vs. −19 % in DH-P, respectively). This difference is probably related to WP being more resistant to pepsin hydrolysis than caseins at pH 3.7 (Kitabatake & Kinekawa, 1998). Similar results have already been observed in our previous work on fermented dairy products (Lavoisier et al., 2023). Furthermore, Gunning and Jacquier (2023) studied the effect of an increase in pH (i.e., from 3, to 3.7, 4.5, or 6) on WP hydrolysis at a pepsin concentration of 2000 U/mL, and they observed that the amount of bioaccessible peptides measured after 2 or 3 h of digestion decreased significantly as pH increased. In contrast, increasing the pH to 3.7 did not have a major effect on casein degradation, either in the present study or in a previous one (Salelles, Floury, & Le Feunteun, 2021). Caseins were digested more rapidly than WP in the gastric phase, and beta-lactoglobulin (β-lg) was particularly resistant to pepsin hydrolysis. This phenomenon was observed in both age groups. A slower rate of alpha-lactalbumin (α-la) hydrolysis was observed in the WP-rich product for older adults, which is again probably related to the decrease in pepsin activity (Lavoisier et al., 2023). In

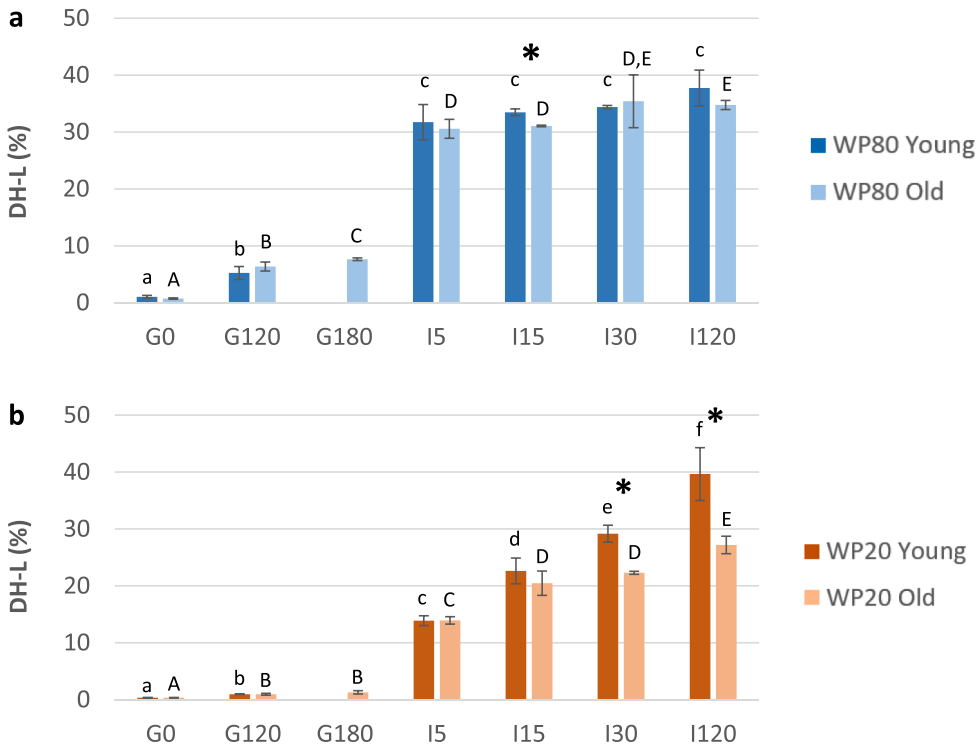

Fig. 6. Degree of lipid hydrolysis (DH-L) during the gastric and intestinal phases of digestion, in young and older adult conditions, in (a) WP-80, and in (b) WP-20. Significant differences ($p \leq 0.05$) according to age are marked with a star (*), and according to the digestion time are marked with different letters (lower case for young adult conditions, and upper case for older adult conditions).

addition, protein particles from both cream cheese samples tended to be larger at pH 3.7 than at pH 3, which could also have influenced the rheological properties of the digesta and/or enzymes diffusion, contributing to the reduction in protein hydrolysis in the gastric phase in older adults.

Protein hydrolysis was also reduced in the intestinal phase for older adults, probably due to the lower concentration of pancreatin (i.e., −20 %) and the lower concentration in bile salts (i.e., –33 %) that are recommended in the simulation of the ageing GIT. The concentration in bile salts is important to consider because they are responsible for destabilizing the globular structure of whey proteins, which facilitates their hydrolysis by pancreatic enzymes (Dulko et al., 2021). A 15 to 20 % reduction in DH-P was measured at the end of the digestion, which is consistent with previous studies comparing the digestion of dairy products by young and older adults (Aalaei et al., 2021a, 2021b; Hernández-Olivas, Muñoz-Pina, Sánchez-García, et al., 2020; Melchior et al., 2023). Some essential amino acids (EAA) were less bio-accessible in older adults than in young adults at the end of digestion, but overall, in this study the effect of age on EAA bioaccessibility was limited. In the presence of bile salts, β-lg was rapidly hydrolyzed by pancreatic enzymes. However, different rates of protein digestion were observed depending on the composition of the products in both age conditions. In WP-80, the DH-P increased constantly during the 120 min of the intestinal phase, but in WP-20, the DH-P had almost reached its maximum after the first minutes of the intestinal phase. Amino acids bioaccessibility followed the same trend, and contrary to expectations, leucine was released faster from the casein-rich product than from the WP-rich product. This is probably related to the offset in WP hydrolysis observed at the end of the gastric phase. Beyond the total amount of hydrolyzed protein, these differences in protein digestion rates are important to consider because it has been shown that amino acids involved in the stimulation of muscle synthesis in older adults, such as leucine, need to be released rapidly during digestion to actually increase protein gain (Dangin et al., 2003). Therefore, it appears that the casein-rich cream cheese formulated in this study may be more adapted to the nutritional needs of older adults than the WP-rich version of the product.

These results contradict most *in vivo* observations where caseins and WP are known as “slow” and “fast” dietary proteins (Boirie et al., 1997). However, they are in line with other *in vitro* studies on milk proteins using a static model of digestion (Egger et al., 2019; Lavoisier et al., 2023; Lorieau et al., 2018), where gastric emptying and continuous secretion of digestive fluids are not simulated. Under static conditions, casein and WP-rich samples remain for the same time in the gastric phase. This may not be the case *in vivo* since gastric restructuring could occur, trapping caseins in the stomach while WP would be rapidly emptied into the duodenum, leading to different digestion kinetics (Mulet-Cabero et al., 2020). The micro and macro-structure of the product may also influence milk proteins digestion, as reviewed by (Fardet, Dupont, Rioux, & Turgeon, 2019). In milk, caseins are found in their micellar form, but in heat-set gels they are aggregated with each other, which could determine their behavior in the stomach and influence their digestion (Le Feunteun et al., 2014).

4.2. Lipid digestion

Lipid hydrolysis in the WP-rich product was not affected by age. It started in the gastric phase and progressed rapidly during the first minutes of the intestinal phase. In this case, neither the decrease in enzyme activities nor the decrease in bile salts concentration affected lipid digestion. In other words, for this type of cream cheese, the conditions recommended to mimic the digestion of healthy older adults over 65 years old were still efficient enough to hydrolyze lipids as well as in young adults unmodified conditions. It shows that the physiological modifications of the GIT related to age may not always be synonymous with a decline in nutrient availability.

In contrast, age had an effect on the hydrolysis of lipids in the casein-rich product. In this type of sample, lipid digestion started in the intestinal phase in both young and older adults. The DH-L then increased

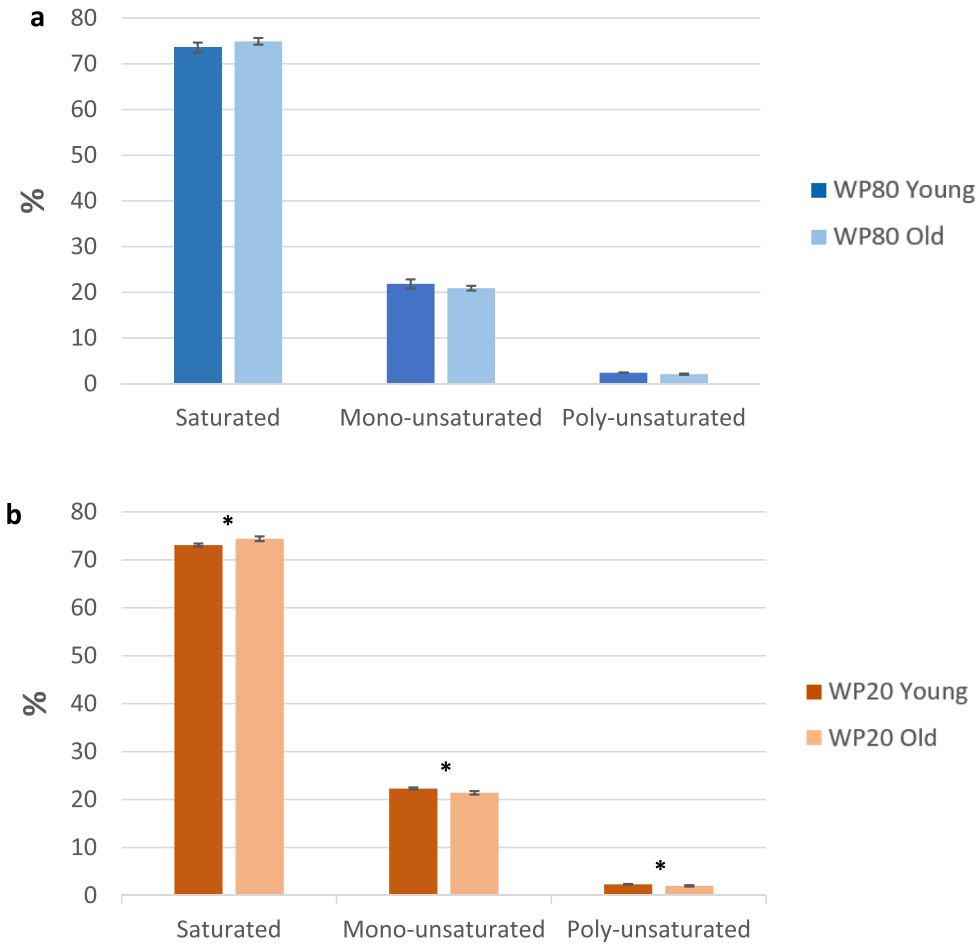

Fig. 7. Proportions of saturated, mono-, and poly-unsaturated fatty acids released at the end of digestion (I120), in young and older adult conditions, in (a) WP-80, and in (b) WP-20. Significant differences ($p \leq 0.05$) according to age are marked with a star (*).

constantly during the 120 min of the intestinal phase, but different kinetics of lipid hydrolysis were observed leading to a significantly lower DH-L (i.e., −38 %), as well as a lower percentage of mono and poly-unsaturated FA released (i.e., −4 and −14 %, respectively) at the end of digestion in older adults. This reduction in lipid hydrolysis in the intestinal phase is attributed to the lower concentration in pancreatic enzymes (i.e., −20 %) and to the lower concentration in bile salts (i.e., –33 %), since both are key parameters involved in the digestibility of fat globules (Asensio-Grau, Peinado, Heredia, & Andrés, 2019; Calvo-Lerma, Fornés-Ferrer, Heredia, & Andrés, 2019).

Contrasting results have been reported in the literature on the *in vitro* digestion of lipids from different foods in older adults. An increase in lipid hydrolysis with age has been observed with fresh and aged cheeses by (Hernández-Olivas, Muñoz-Pina, Sánchez-García, et al., 2020), but no effect of age conditions was reported with different fish species in another study from the same team (Hernández-Olivas, Muñoz-Pina, Andrés, & Heredia, 2020). On the other hand, a delay in lipid hydrolysis for older adults was observed in pork sausages (Peyron et al., 2021), and vegetable oil emulsions (Wang, Shi, Xu, Tan, & Liu, 2024).

The differences in lipid digestion observed between WP-80 and WP-20 are probably related to their microstructure under the acidic conditions of the simulated gastric phase. At pH 3 and 3.7, smaller lipid droplets were observed in WP-80 than in WP-20, which is known to improve lipid digestibility (Asensio-Grau et al., 2019; Calvo-Lerma et al., 2019; Drouin-Chartier et al., 2017). This may be due to the process used to manufacture the WP-rich cream cheese (i.e., including a homogenization step), and/or to the emulsifying properties of WP (Yamauchi, Shimizu, & Kamiya, 1980) reducing droplet coalescence during digestion. Casein aggregation also occurred at pH < 4.6. This effect was limited in WP-80 and resulted in the formation of small protein aggregates. However, in WP-20 a weak gel formed and large protein particles were observed at pH 3 and 3.7. Lipid droplets may have been trapped in these casein-rich particles, making them less accessible for the gastric lipase. This structure-related effect has already been previously reported in studies on matrices containing proteins and lipids (Calvo-Lerma, Fornés-Ferrer, Heredia, & Andrés, 2018; Guo, Ye, Bellissimo, Singh, & Rousseau, 2017; Mulet-Cabero et al., 2020). As a result, in the WP-rich cream cheese, gastric lipase was able to initiate lipid digestion in the gastric phase, even if its activity was reduced, and its products then probably facilitated further lipid hydrolysis by pancreatic lipase in the intestinal phase. On the contrary, the microstructure of the casein-rich cream cheese was more challenging for the gastric lipase, and lipid digestion was then hindered by older adult sub-optimal conditions in the intestinal phase.

In conclusion, in this study we observed a significant effect of age on the digestion of high-protein cream cheese samples. However, this effect depended on the ratio of caseins to WP in the product, which influenced its susceptibility to enzymatic attack. At the end of digestion, protein hydrolysis was reduced in both samples in digestive conditions relevant to the physiology of older adults, but lipid hydrolysis was higher in the WP-rich sample than in the casein-rich sample. Based on the results of this study, protein digestion seems to decrease with age, and therefore increasing the quantity of high-quality proteins in older adults' diets is probably necessary to avoid malnutrition. In terms of muscular health, casein-rich products could be more adapted to the specific needs of older adults than equivalent WP-rich products, as it appears that caseins are

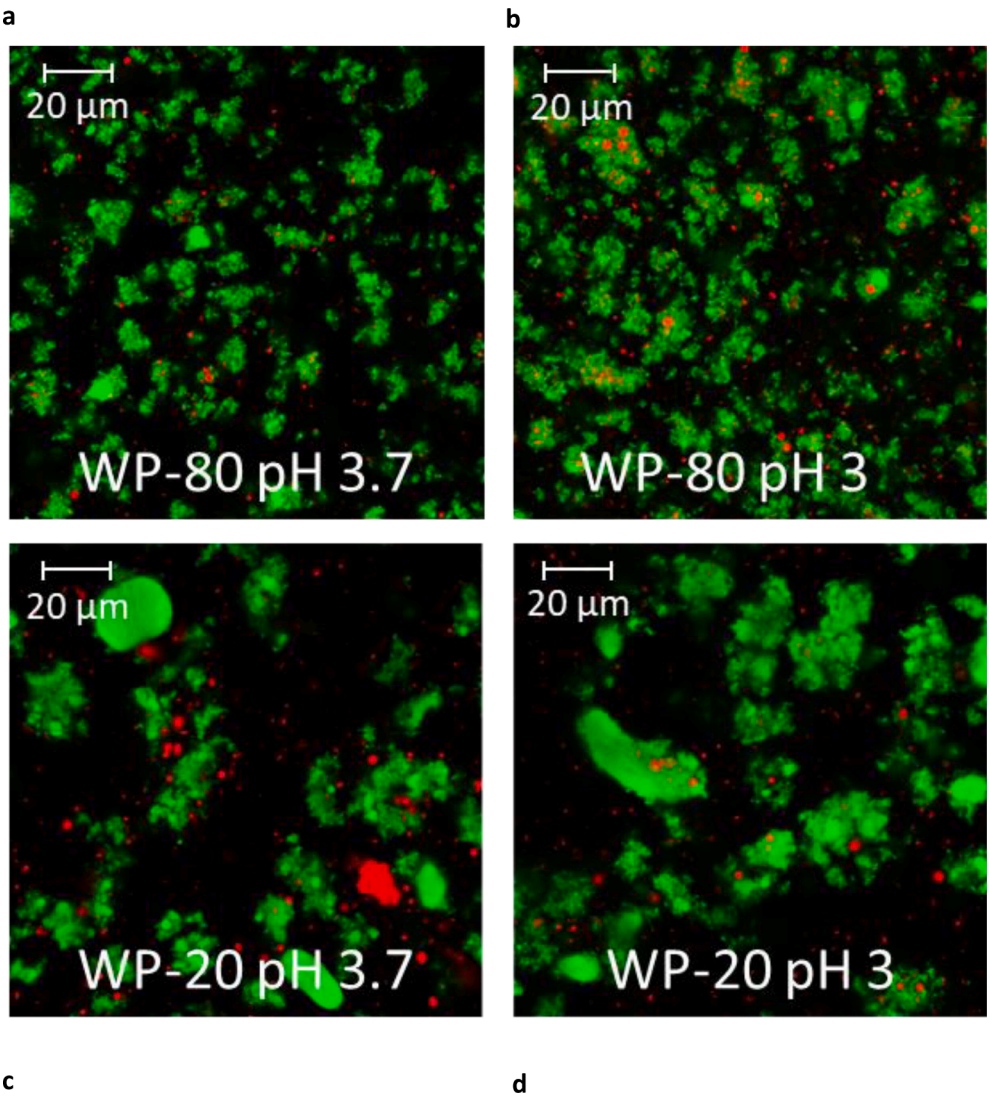

Fig. 8. Confocal microscopy images of WP-80 and WP-20 at pH 3.7 (older adult conditions in the gastric phase,) and at pH 3 (young adult conditions in the gastric phase). Lipid droplets appear in red and protein aggregates in green. Images are representative of the three repetitions measured for each condition.

hydrolyzed more easily than WP in sub-optimal digestion conditions. However, this may not be always true as milk proteins hydrolysis will probably vary significantly according to the microstructure of the dairy product. Concerning lipids, the results of this study suggest that age does not always impact lipid hydrolysis. Nutritional recommendations concerning lipids could therefore be the same for young and older adults from a digestive point of view. The impact of product composition and microstructure on lipids digestibility is an interesting outcome of this study and it should be further studied (e.g., in other types of food matrices). However, in general, observations from this *in vitro* study should be first confirmed *in vivo* by clinical studies before being used to develop food products for the elderly.

CRediT authorship contribution statement

Anaïs Lavoisier: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Séverine Chevalier:** Data curation, Investigation, Methodology. **Gwénaële Henry:** Data curation, Investigation, Methodology. **Jordane Ossemond:** Data curation, Investigation, Methodology. **Marielle Harel-Oger:** Methodology, Resources. **Gilles Garric:** Methodology, Resources. **Didier Dupont:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Martine Morzel:** Conceptualization, Formal analysis, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114621.

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