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Impact of process and composition of formulas for elderly on *in vitro* digestion using the dynamic DIDGI® model

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ABSTRACT

Due to the lower efficiency of the elderly digestion system, new formulations are needed in order to increase the bioaccessibility of macronutrients. The aim of the work was to evaluate the effect of the process of protein sources production using either liquid (F2) vs spray dried milk proteins (F1/F3) and the source of lipids (vegetable oil (F1) vs mix of vegetable oil + bovine milk cream (F2/F3)) ingredients on the macronutrient digestion of three experimental elderly formulas. The dynamic *in vitro* digestion model DIDGI®' was adapted to simulate the digestive conditions of the elderly. An exhaustive review of the literature was carried out in order to simulate as closely as possible the elderly digestive parameters and constituted the starting point towards a consensus *in vitro* digestion model that will be proposed soon by the INFOGEST scientific network. The three experimental formulas (F1/F2/F3) differing by the composition and process applied were submitted to the DIDGI® dynamic *in vitro* digestion over four hours using parameters adapted to the elderly. The three formulas were compared in terms of proteolysis and lipolysis. A slight impact of the process (liquid vs spray-dried) on the degree of proteolysis at the end of digestion was observed with 50.8% for F2 compared to 56.8% for F1 and 52.9% for F3 with<5% of difference between the 3 formulas. Concerning the degree of lipolysis, the addition of bovine cream led to a lesser extent of lipolysis with 63.7 and 60.2% for F2 and F3 respectively versus 66.3% for F1 (containing only vegetable oil). Our results highlighted the beneficial input of the milk fat with a higher level of phospholipids and a lower ω6/ω3 PUFA ratio and can be a good alternative to the use of the vegetable fat in drinks for elderly people.

1. Introduction

Ageing has a strong effect on gastrointestinal digestion (Rémond et al., 2015). The decrease in digestion and absorption efficiency can contribute to the risk of malnutrition. In Europe, a prevalence of malnutrition of 28% in the older population at hospital, is observed, against 17.5% in residential care and 8.5% in the community (Leij-Halfwerk et al., 2019). Malnutrition, together with the impaired properties of the digestion tract during ageing, can contribute to the increase of sarcopenia i.e. muscle loss in the elderly population (Aragon & Schoenfeld, 2013; Granic et al., 2019; Rashid et al., 2020). In order to limit sarcopenia in the elderly population, the nutritional protein intake for this population must be higher than that of adults (1–1.5 vs 0.8 g/ kg of body weight/day) (Nowson & O'Connell, 2015). The amino acid composition of the proteins has an important role on muscle synthesis, notably the leucine (Ham et al., 2014; Katsanos et al., 2006).

For the elderly population, milk is a good source of essential amino acids (Zanini et al., 2020) due to their high digestible indispensable amino acid score and amount of essential amino acids per gram of protein (Wolfe, 2015). Furthermore, milk proteins are characterized by 95% of true digestibility and 74% of net postprandial protein utilization (Bos et al., 1999; Morens et al., 2003). The heat treatment can play an important role in the protein digestion and digestibility (van Lieshout et al., 2020). Denaturation and aggregation that take place after heat treatment can enhance or reduce the milk protein digestibility, respectively (Wada & Lönnerdal, 2014, 2015). The digestion of β-lactoglobulin

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is higher when strong pasteurization and sterilization processes take place than low-temperature pasteurization processes (Loveday et al., 2014; Peram et al., 2013). Conversely, caseins and α-lactalbumin were hydrolyzed independently of heat treatments (Tunick et al., 2016).

Unlike with proteins, no recommendations on lipid consumption are established, although the consumption of foods with higher polyunsaturated and saturated fatty acids were associated with a lower loss of the cognitive performance and lower hypertension, respectively (González et al., 2010; Nakamura et al., 2019). In milk, fat is organized in complex physicochemical structures called milk fat globules. These globules are composed of a tri-layer outer membrane –consisting of a double layer of phospholipids (PLs) placed on an inner PLs monolayer – along with a triacylglycerol core (Lopez et al., 2015). The milk fat globules present different bioactive properties. Several animal and human studies have provided evidence that the consumption of PLs delayed the ageing of skeletal muscle and contribute in several bioactivities such as the brain development, the cognitive functions and the immunity development (Abd El-Salam & El-Shibiny, 2020).

Different *in vitro* digestion models have been designed to simulate the elderly digestive conditions and have been recently reviewed by Makran et al considering either static or dynamic *in vitro* digestion models applied to study nutrient digestibility (Makran et al., 2022). Levi & Lesmes, 2014 were the first to develop a bi-compartmental model; they investigated the digestion of whey proteins and a commercial liquid drink (Shani Levi et al., 2017). The second model developed was an adaptation of the TNO Gastro-Intestinal Model (TIM) to simulate the digestion of meat (Denis, Sayd, Georges, Chambon, Chalancon, Santé-Lhoutellier, et al., 2016) in the elderly. Recently, a dynamic model simulating the biomechanical contractions of the stomach has been used to investigate the digestibility of apple polyphenolics (Shang et al., 2022) and a new model was designed to simulate the colon fermentation into the gut (S. Y. Lee et al., 2021).

Through a comprehensive review of the literature on the digestive conditions in the elderly, key parameters were defined. The important changes reported were the lower gastric (Feldman et al., 1996; Moreau et al., 1988) and intestinal (Laugier et al., 1991; Vellas et al., 1988) enzyme activities than in younger adults, together with a higher intragastric pH (Russell et al., 1993), a slower gastric emptying (Clarkston et al., 1997; Moore et al., 1983; Nakae et al., 1999) and a lower quantity of bile salts (Salemans et al., 1993).

The model proposed and applied in the present study considered those digestive changes as compared to the adult and will be a valuable tool for assessing the nutritional properties of food for the elderly in the near future and is a first step towards a consensus *in vitro* digestion model that will be defined by the INFOGEST scientific community.

Due to lower digestion efficiency, development of new formulas leading to macronutrient bioaccessibility improvement is needed to address the increase in the elderly population in recent decades. The aim of the work was to design three high protein milk formulas (F1/F2/F3) for the elderly population and to evaluate the effect of the process (liquid vs spray dried milk protein isolate) and lipid source (vegetable oils vs a mix of vegetable oil and bovine cream) on the kinetics of protein and lipid digestion. To achieve this, the DIDGI® *in vitro* dynamic model was set up in order to simulate the *in vivo* elderly digestive conditions.

2. Materials and methods

All chemicals and enzymes were purchased from Sigma Aldrich (Saint Quentin Fallavier, France), unless otherwise stated. Rabbit gastric extract (RGE) was provided by Lipolytech (Marseille, France).

2.1. Design of formulas for elderly

Briefly, three formulas (F1, F2 and F3) fulfilling the nutritional needs of elderly people were developed in the present study. They differed according to the nature of their protein ingredient and/or the nature of fat. F1 was manufactured using spray-dried milk protein concentrate (MPC) and a mixture of 60% rapeseed and 40%sunflower oil. In F2, spray-dried MPC was replaced by liquid MPC and sunflower oil by milk fat. Finally, F3 was made of spray-dried MPC and the same fat as F2.

2.1.1. Milk protein ingredient

Raw milk from grass-fed cows was received into a commercial dairy processing plant (Glanbia Ireland, Ballyragget, Kilkenny, Ireland), the fat was removed by centrifugal separation and the resulting skimmed milk (~8% solids) was pasteurized (72 °C × 15 sec). This milk was then concentrated to 20% total solids by spiral membrane ultrafiltration and either used with no further processing, as liquid milk concentrate, or further concentrated through evaporation and then spray dried to produce milk protein isolate powder (minimum protein content 85%).

2.1.2. Formula manufacture

Milk protein isolate powder was reconstituted in water at 50 °C for 30 min under vacuum high shear conditions, to achieve 10% protein (w/v) (F1 and F3). Liquid milk concentrate was mixed with water at room temperature (20–25 °C) for 10 min to achieve 10% protein (w/v) (F2). A carbohydrate source, composed of sucrose and maltodextrin (DE15), was rehydrated with water at room temperature by mixing under high shear for 15 min. The carbohydrate source was added to the 10% protein solutions and mixed under high shear. Vitamins and mineral salts were added under mixing to ensure that the nutritional profile of the prototypes complied with UK dietary reference values (Salmon, 1991). The mixture was preheated to 55 °C for lipid addition. The lipid fraction, which was composed by 60% of sunflower oil and 40% of either rapeseed oil (F1) or bovine cream (F2 and F3), was preheated in the presence of emulsifiers, mixed under higher shear and then added to the mixture with further high shear mixing. The mixtures were pre-heated (80 °C × 120 sec) before being ultra-heat treated by direct steam infusion, followed by aseptic homogenisation at 75 °C (250 bar), cooled to ambient and aseptically filled into sterile HDPE bottles with foil lid.

2.2. Composition of elderly formula

Protein and lipid composition of the formula are given in Table 1.

2.2.1. Protein content

Total protein, non-protein nitrogen (NPN) and non-casein nitrogen (NCN) were determined by the Kjeldahl's method according to ISO standard 8968–1 (International Dairy Federation, 2014) with a conversion factor of 6.22 for the total protein nitrogen (calculated by the amino acids composition of the formulas), 6.19 for the NPN and 6.38 for the NCN.

2.2.2. Lipid content

Total fatty acids (TFAs) of the formulas were quantified as described by Nebbia et al. (2022). After methylation, TFAs were quantified by using a GC–MS (Shimadzu GCMS-TableQP2010 SE, Shimadzu Corp., Japan) equipped with a BPX70 capillary column (120 m, 0.25 mm i. d., 0.25 μm film; SGE Analytical Science, Australia) as previously described (Furtado et al., 2021).

2.2.3. Phospholipids content

Phospholipids (PLs) extraction: 500 μL of formulas were mixed with 10 ml of chloroform–methanol mixture (2:1, v:v) and 20 μL of phosphatidylglycerol (5 mg/mL) as internal standard. After shaking (1 h), samples were washed with KCl 0.8% and chloroform–methanol-KCl 0.8% (3:48:47, v:v:v). After filtration (Whatman filter paper, 2.5 μm, Grosseron, France), lipids were evaporated under nitrogen. Lipid samples (30 mg) were solubilized in 500 μL of chloroform–methanol (9:1, v/v) and PLs were extracted using a silica gel bonded column (Supelclean LC-SI, 6-ml volume, 1 g sorbents, Supelco Bellefonte, USA) as described previously by Avalli & Contarini (2005). Briefly, after conditioning with

Table 1
Protein and lipid composition of the formula. Letters indicated the differences between samples.

	F1	F2	F3
Protein ingredient	Milk protein isolate powder	Liquid milk concentrate	Milk protein isolate powder
Protein (g/100 ml)	9.62 ± 0.21[a]	9.4 ± 0.01 [a]	9.4 ± 0.13 [a]
Casein: whey protein ratio	80:20	80:20	80:20
Non Protein Nitrogen(g/100 ml)	0.16 ± 0.02[a]	0.23 ± 0.01[b]	0.15 ± 0.02[a]
Non Casein Nitrogen(g/100 ml)	0.43 ± 0.02 [a]	0.75 ± 0.01[b]	0.41 ± 0.01[a]
% Essential Amino Acids	42.99 ± 0.15[a]	43.07 ± 0.14[a]	42.96 ± 0.09[a]
% Non essential Amino Acids	57.01 ± 0.15[a]	56.93 ± 0.14[a]	57.04 ± 0.09[a]
Lipid ingredient	Rapeseed oil (60%) sunflower oil (40%)	Rapeseed oil (60%) bovine cream (40%)	Rapeseed oil (60%) bovine cream (40%)
Lipids (g/100 ml)	5.12 ± 0.11[a]	4.76 ± 0.1[a]	5.09 ± 0.11[a]
% Saturated FAs	10.39 ± 0.12[a]	32.44 ± 0.03[b]	31.66 ± 1.06[b]
% Mono-unsaturated FAs	69.48 ± 0.16[a]	48.16 ± 0.03[b]	47.27 ± 0.27[b]
% Poly-unsaturated FAs	20.13 ± 0.04[a]	19.4 ± 0.01[b]	21.07 ± 0.87[c]
Ratio ω6/ω3	3.92 ± 0.01[a]	2.51 ± 0.03[b]	2.54 ± 0.02[b]
$mg_{Phospholipid}/ml_{formula}$	0.89 ± 0.02[a]	1.51 ± 0.1[b]	1.62 ± 0.1[b]
$mg_{Phospholipid}/100\ mg_{lipid}$	1.51 ± 0.13[a]	2.92 ± 0.02[b]	2.51 ± 0.47[b]
% Phosphatidylinositol	32.15 ± 0.88[a]	23.77 ± 1.34[b]	20.69 ± 0.65[c]
% Phosphatidylethanolamine	20.95 ± 0.09[a]	26.67 ± 0.61[b]	28.23 ± 1.66[b]
% Phosphatidylcholine	41.04 ± 0.24[a]	46.06 ± 2.14[b]	47.52 ± 0.91[b]
% Sphingomyelin	5.86 ± 0.72[a]	3.5 ± 0.19[b]	3.55 ± 0.32[b]

10 ml of chloroform–methanol (2:1, v/v), non-polar lipids were eluted with 6 ml of hexane–diethyl-ether (8:2, v/v) and 6 ml of hexane–diethyl-ether (1:1, v/v). PLs were collected with 4 ml of methanol and 4 ml of chloroform–methanol–water (3:5:2, v/v/v). Samples were evaporated under nitrogen and solubilized in 250 μL of chloroform–methanol (9:1, v/v) before HPLC injection.

HPLC separation and quantification: PLs were separated as described previously by Braun et al. (2010) using two Nucleosil 50–5, 250́ 3 mm, 5 mm columns equipped with a precolumn, Nucleosil 50–5, 8́ 3 mm, 5 mm (Macherey-Nagel, Easton, PA) with an Agilent Technologies 1100 module and an inline PL-ELS 1000 ELSD. PLs were separated using two solvents (A (3 g/L of ammonium formiate) and B (acetonitrile/methanol (100/3 v/v)) and gradients were from 1% to 30% of solvent A from 0 to 19 min, stable to 30% solvent A until 21 min and restored to initial condition until minute 24. The flow rate was 1 ml/min and the separation was performed at 55 °C. The ELSD condition were: 90 °C for the evaporation, 40 °C for the nebulization and compressed air with a flow rate of 1 L/min. PLs standards were used for the PLs identification and quantification and peak area measurements were compared against the internal standards.

2.3. Elderly dynamic in vitro digestion model

Formulas were submitted to gastrointestinal digestion using the bi-compartmental *in vitro* dynamic system DIDGI® (Ménard et al., 2014). The system was set up to simulate the digestive conditions of the elderly population over 65 years old. The specific gastrointestinal parameters were summarized in Table 2. Gastric and intestinal simulated fluids were set as an adult as described (Lindahl et al., 1997). The fasted gastric conditions were simulated as described for adults (Egger et al., 2019) due to a lack of literature data, i.e. with 24 ml of gastric fluids at pH 2.2.

Gastric and intestinal emptying followed the Elashoff's equation (Elashoff et al., 1982). Gastric emptying half-time was set up at 86 min and the shape of the curve at β = 1 as determined *in vivo* by Moore et al. (1983). For the intestinal emptying ($t_{1/2}$ = 160 and β = 1.6), values were the same as those used by Minekus et al. (1995) due to the similar intestinal transition time between adult and elderly population (Rémond et al., 2015). The acidification curve in the gastric compartment was designed in order to simulate the *in vivo* data as described by Russell et al. (1993) and followed the equation: pH acidification = $5.6111e^{-0{,}005*time}$. The intestinal pH was fixed and constant at 6.5 (Russell et al., 1993).

Table 2
Gastrointestinal parameters for *in vitro* dynamic digestion of elderly drinks simulating elderly conditions. SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

Component		Value	References
Gastric conditions (37 °C)			
Fasted state	SGF	24 ml	Egger et al. (2019)
	pH	2,2	
Milk ingested	Total volume	100 ml	
Gastric pH	Acidification curve	pH = 5,6111e-0,005*time	Russell et al. (1993)
SGF + enzymes	Pepsin	1200 U/mL of gastric content	Feldman et al. (1996)
	Gastric lipase	9 U/mL of gastric content	Moreau et al. (1988)
	Flow rate	0,25 ml/min (Pepsin)	
		0,25 ml/min (Lipase)	
Gastric emptying (Elashoff fitting)	$t_{1/2}$	86 min	Moore et al. (1983)
	β	1	
Intestinal conditions (37 °C)			
Intestinal pH		6,5	Russell et al. (1993)
SIF + pancreatin	Pancreatin	5,5 % solution	Laugier et al. (1991) and Vellas et al. (1988)
	Flow rate	0,25 from 0 to 240 ml/min	
SIF + bile	Bile	2,7 % solution	Salemans et al. (1993)
	Flow rate	0,5 fom 0 to 30 ml/min	
	Bile	1,3 % solution	
	Flow rate	0,5 fom 30 to 240 ml/min	
Intestinal emptying (Elashoff fitting)	$t_{1/2}$	160 min	Rémond et al. (2015)
	β	1,6	

In the gastric phase, pepsin and lipase amounts were calculated from data obtained in previous *in vivo* studies in order to simulate a loss of 40% for pepsin (Feldman et al., 1996) and 85% for lipase (Moreau et al., 1988) as compared to the adult values reported by Egger et al. (2019). Rabbit gastric extract (RGE) was used to cover the gastric lipase units (9 U/mL of gastric content) while a supplementation with porcine pepsin was needed to reach the 1200U/ml of gastric content. Intestinal enzymes were added as porcine pancreatin. The quantity of pancreatin was 20% less than the adult one and based on the coverage of the lipase units needed as determined in the *in vivo* studies of Laugier et al. (1991) and Vellas et al. (1988). The pancreatin solution (5.5%), with a flow rate of 0.25 ml of pancreatin solution/min, allowed to reach 88 U/min of trypsin and 852.5 U/min of intestinal lipase during intestinal digestion. Bovine bile salts were 33% less of those of the adult (Salemans et al., 1993).

Dynamic digestions were performed in triplicate over 4 h. Aliquots were collected before digestion (G0) and at 45, 90, 135, 180 and 240 min after formula ingestion. in both gastric and intestinal compartments. The particle size measurements were performed immediately after sampling. Direct lipid extraction was performed after the addition of lipase inhibitor (50 μL of 0.1 M 4-bromophenylboronic acid per mL of digesta). In order to inhibit pepsin, 50 μL of pepstatin A (0.72 mM)/ml of gastric digesta and for trypsin inhibition, 50 μL of pefabloc (0.1 M)/ml of intestinal digesta were added before storage at −20 °C.

The dilution of the digestive samples during digestion was evaluated by the quantification of the norleucine added as an internal marker to the formulas with a final concentration of 1.5 mg/ml before digestion. The dilution factors were calculated with the ratio between the amount of Norleucine quantified at each digestion time divided by the amount of Norleucine before digestion.

2.4. Determination of the degree of lipolysis (DL)

Lipids were extracted from digesta samples according to the Folch's method as described by Nebbia et al. (2022). After methylation, TFAs were quantified by using a GC–MS as described by (Furtado et al., 2021). The % of DL was calculated as followed:

$$DL\,(\%) = 100\,x\,\frac{[FFA(t)]\,x\,S}{[TFA]}$$

Where [FFA(t)] was the FFAs detected at each digestion time (μmol/L of digesta) and S was the estimated ratio of undigested formulas within the digesta (L of meal/L of digesta) based on the norleucine quantification.

2.5. Determination of the degree of proteolysis (DP)

2.5.1. Free-amine release

The overall DP was calculated from the measurement of primary amines (–NH_2) using the O-phtaldialdehyde (OPA) method. After centrifugation (10000xg for 20 min at 4 °C), 50 μL of supernatant were mixed with 100 μL of the OPA reagent (0.5% w/v SDS, 0.25 mg/mL OPA, 7 mM DTT, 20 mM sodium tetraborate) in a 96-well plate and the absorbance at 340 nm was read after 10 min at 37 °C. The DP was calculated as follows:

$$DP\,(\%) = 100\,x\,\frac{([NH_2(t)] - [NH_2(secretions)])\,x\,1/S - [NH_2(t0)]}{[NH_2(total)] - [NH_2(t0)]}$$

Where [NH_2(t)] was the mg NH_2/L digesta of the primary amines quantified at each digestion time from which were removed the [NH_2] of the secretions. S was the estimated ratio of undigested formulas within the digesta (L of meal/L of digesta). [NH_2(t0)] was the concentration of primary amines present in the meal before digestion and [NH_2 (total)] was the total primary amines and was determined in each formula before digestion, following total acid hydrolysis in 6 N HCl at 110 °C for 24 h.

2.5.2. Protein profile analysis by electrophoresis

Proteins were analysed by SDS-page using 4–12% bis tris gels loading 6 μg of protein/well as described by Nebbia et al. (2022).

2.5.3. Amino acid quantification during digestion

During intestinal digestion, AAs were quantified after sulfosalicylic acid precipitation by a cation exchange chromatography on a Biochrom 30 automatic AA analyzer (Biochrom Ltd., Cambridge, G.B.) as previously described (de Oliveira et al., 2016). The % bioaccessibility of the AAs was calculated as follow:

$$AA\ bioaccessibility\,(\%) = 100\,x\,\frac{[AA(t)] - [AA\,(secretions)]}{[AA(total)]\,x\,S}$$

where [AA(t)] was the concentration of free AAs at each digestion time and [AA(secretions)] was the concentration of AA from bile and pancreatin (mg/L of intestinal digesta). The total amount of each AA [AA(total)] was obtained after 6 N HCl hydrolysis of the undigested formulas at 110 °C for 24 h. Cysteine and methionine were measured as methionine sulphone and cysteic acid after performic oxidation. S was the estimated ratio of undigested formulas within the digesta (L of meal/L of digesta). Cysteine was under the detection limit in the free AAs formulas and no tryptophan was quantified in total AAs formulas.

2.6. Particle size distribution

The distribution of the particle size before and during gastric digestion was determined using a Mastersizer 2000 laser light scattering (Malvern Instruments, Malvern, UK). The refractive indexes used were 1.45 for lipid at 633 and 466 nm and 1.33 for water (dispersant solution in the measurement cell). The diameter mode and the diameter mean D [4,3] were calculated and determined with or without sodium dodecyl sulfate (SDS). Results obtained on particle size have not been presented separately in the present study but used in the integrated overall analysis.

2.7. Statistical analysis

Data analyses were performed using the R software, version 4.1.3. In the undigested samples formulas, statistical analyses were performed using one-way ANOVA, with formulas type as fixed factor. During digestion, a two-way ANOVA was used separately on gastric and on intestinal values with time and type of formulas set as fixed factors. Residual normality and variance homogeneity were tested for each variable using a Shapiro-Wilks and Levene's test, respectively. Where significance was assessed (p value < 0.05), pairwise multiple comparison of the means was carried out using Tukey's test. Principal Component Analysis (PCA) was performed using the factoextra package. Individuals were divided in gastric (G180, G240) and intestinal (I90, I135, I180, I240) phases. The variables considered in the PCA were: hydrolysis and lipolysis degree (HD and LD), relative % of free saturated, mono- and polyunsaturated fatty acids, particle size distribution characteristics in the gastric phase (D[4,3]-diameter), AA bioaccessibility and relative % of free essential and non-essential AAs in the intestinal phase.

3. Results and discussion

The objective of the present study was to compare the digestion kinetics of three protein-rich formulas in terms of proteolysis and lipolysis and to study the impact of the process applied on the protein fraction (liquid or spray-dried) and the impact of the addition of bovine cream in the lipid fraction.

3.1. Elderly formulas characterization

The present study has evaluated whether the nature of lipids (vegetable oil vs bovine cream) and the process used to generate protein ingredients (spray-dried vs liquid) could modify the kinetics of digestion of lipids and proteins and the bioaccessibility of free AAs and free fatty acids in formulas designed for the elderly Formulas containing high protein level (10%) composed by liquid milk concentrate (F2) or milk protein isolate powder (F1 and F3), vegetable oil (F1) or bovine fat (F2 and F3). The NPN constituted only 2% of the total nitrogen for the 3 formulas and for the NCN value of F2 was 1.8 times higher than for F1 and F3 (Table 1). By the SDS-PAGE under non-reducing conditions, formulas exhibited a similar protein profile with bands at higher molecular weight, in particular between 55 and 66 kDa (Supplementary data Fig. 1). The AAs profiles were quite similar between samples, glutamate and glutamine were the major AAs (21.4 %) followed by proline (9.8%), leucine (9.5%) and lysine (7.9%) (Supplementary data Fig. 2). Due to the different sources of lipids in the formulas, the saturated and unsaturated profiles were different between formulas with higher quantity of saturated fatty acids in - F2 and F3 containing milk fat (Table 1). The fatty acid composition showed significant differences in the proportion of the most abundant fatty acids, with F2 and F3 sharing a similar fatty acid profile than the F1 sample (Supplementary data Fig. 3). The fatty acid profile was dominated by the oleic (C18:1n9c), linoleic (C18:2n6c) and palmitic (C16:0) acids. The differences in fatty acids composition led to a significant different ω6/ω3 PUFA ratio with the formulas F2 and F3 that had the lower ratio (2.5). F2 and F3 formulas contained 1.8 times higher PLs present on the surface of the droplet of the bovine cream than sample F1. PC was the major PL class in all samples. The PLs profile was quite similar in the two formulas with the bovine cream with higher % of PE and lower quantity of PI and SM compared to the sample F1 (Table 1). Finally, formulas had a similar particle size distribution of 0.17 μm (Supplementary data Fig. 4).

3.2. Lipolysis

In the gastric phase, the DL was statistically different between samples, and increased more slowly for F3 (6.2%) and rapidly for F1 (13.2%) (Fig. 1A). The FFAs profiles were strongly different between samples. More than 65% of the F2 and F3 profiles were constituted by saturated fatty acids while, for the other samples, the profiles were constituted primarily by mono-unsaturated fatty acids. F2 and F3 had a significantly higher quantity of C6 to C16 fatty acid forms and a significantly lower % of the C18:1n9c and C18:2n6c fatty acids (Fig. 1B). In the intestinal phase, around 50% of the free fatty acids were released after 90 min of digestion and the DL increased to reach 66.3 ± 4.9%, 63.7 ± 4.4% and 60.2 ± 3.5 % at the end of the intestinal phase for F1, F2 and F3 respectively with a slightly significant difference between F1 and F3 ($p < 0.05$ greater than 0.01) (Fig. 1A). As already described for the gastric phase, the FFAs profiles were strongly different between formulas (Fig. 1C). The fatty acids profiles of the F2 and F3 formulas were constituted for more than 36% of saturated fatty acids while in the other formulas the saturated fatty acid represented 17% of the total lipid profile. Interestingly, during digestion, differences were found in the release of free fatty acids between the gastric and the intestinal phases. The fatty acid profiles of F2 and F3 containing bovine cream had higher mono-unsaturated fatty acids in the intestinal phase than the gastric phase (50% vs 25%). The short chain FAs (C6:0 to C14:0) were released

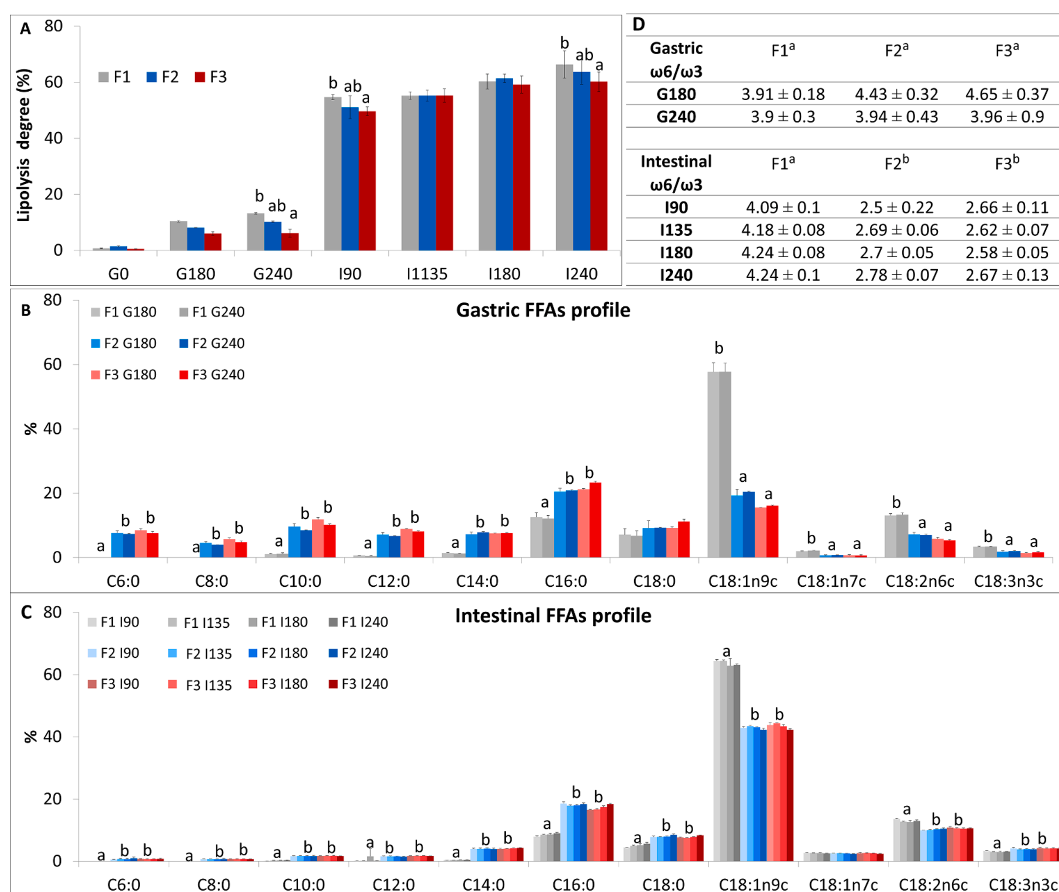

Fig. 1. Degree of lipolysis of the formulas (A), their fatty acids profiles during gastric (G180 and G240 min) (B) and intestinal (I90-I135-I180-I240 min) (C) digestion and the ω6/ω3 free PUFA ratio of samples during digestion (D). The data represent means ± standard deviations (n = 3). Tukey's post hoc tests were performed and letters on the graph indicate different classes.

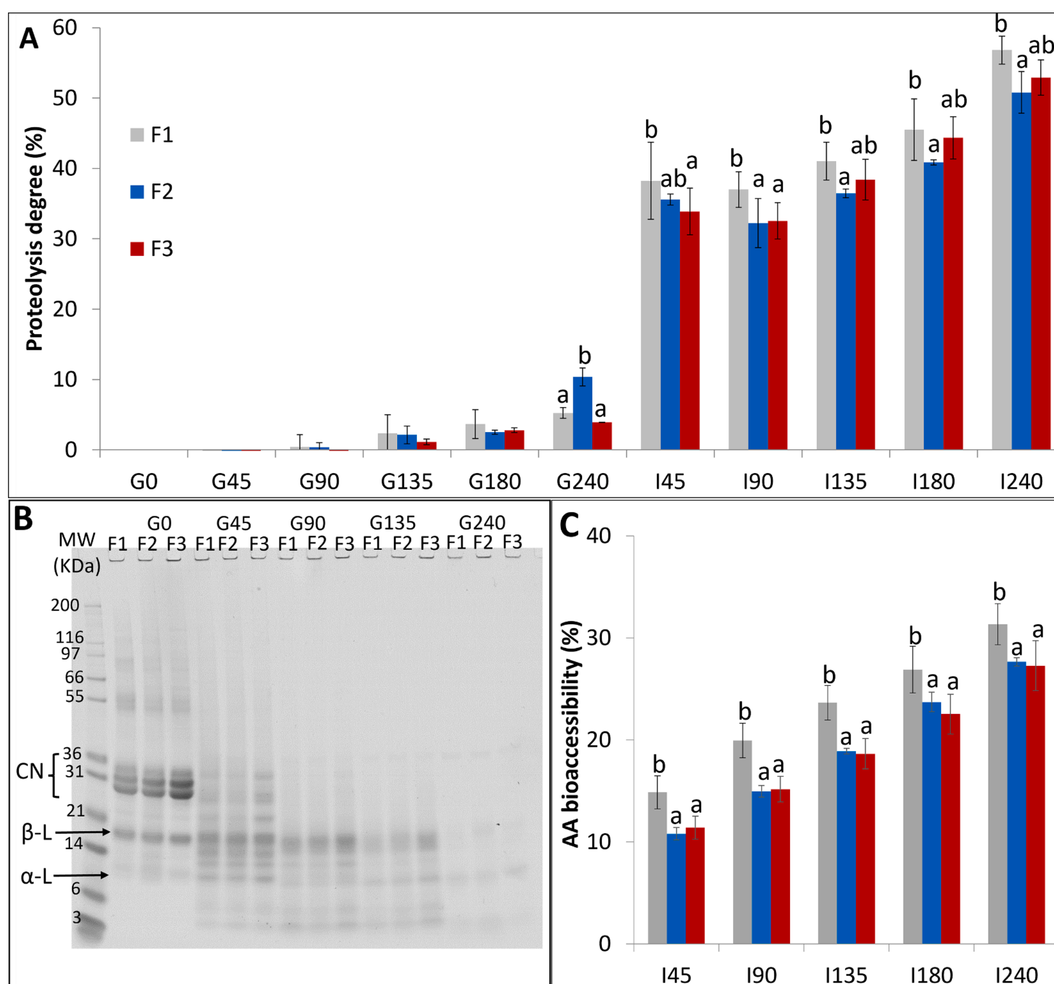

Fig. 2. Degree of protein hydrolysis of the digesta samples during gastric (G0-G45-G90-G135-G180-G240 min) and intestinal (I45-I90-I135-I180-I240 min) digestion (A). Protein profile of the samples during gastric digestion (B). Proteins were loaded on the gels taking into account the dilution with the gastric and intestinal juices at different sampling times. CN: casein; β-L: β-lactoglobulin; α-L: α-lactalbumin. Amino acids bioaccessibility of the digested samples during intestinal digestion (C). The data represent means ± standard deviations (n = 3). Tukey's post hoc tests were performed and letters on the graph indicate different classes.

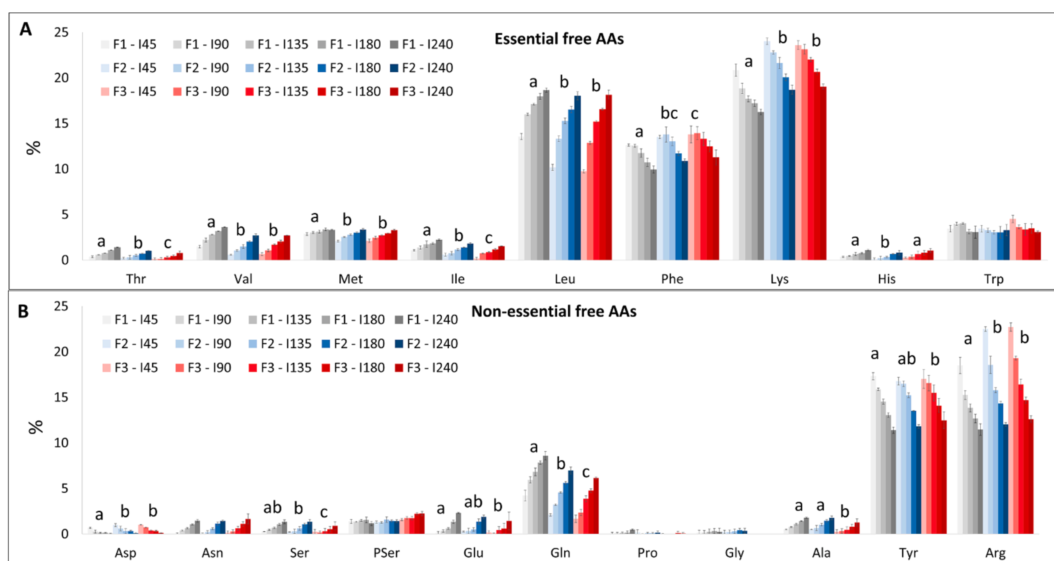

Fig. 3. Essential (A) and non-essential (B) amino acid profiles during intestinal digestion (I45-I90-I135-I180-I240 min). Tukey's post hoc tests were performed and letters indicate the different classes.

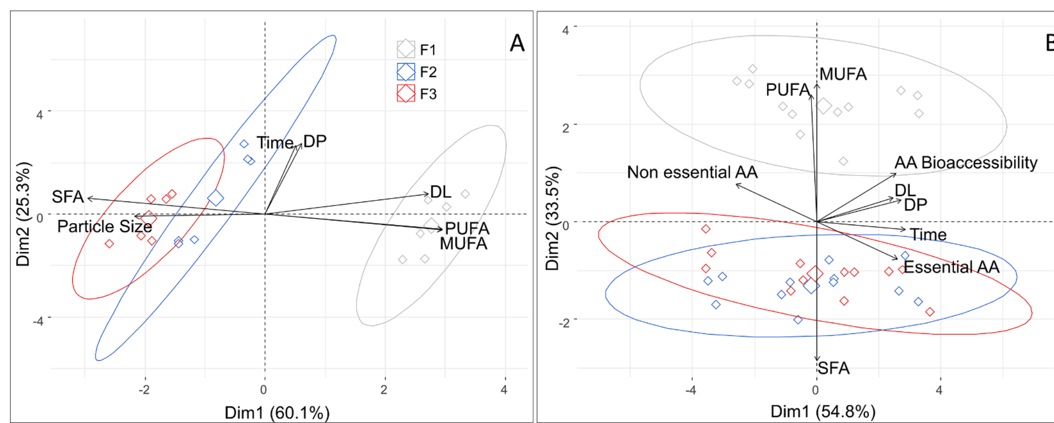

Fig. 4. Projection of the variables and the individuals on the first and second dimensions (Dim1 and Dim2) of the principal component analysis (PCA) of the digested samples during gastric (G180-G240 min) digestion (A) and intestinal (I90-I135-I180-I240 min) digestion (B). Parameters: degree of proteolysis and lipolysis (DP and DL); relative % of free saturated, mono- and poly- unsaturated fatty acids (SFA, MUFA and PUFA); particle size distribution characteristics in the gastric phase (D [4,3]-diameter); amino acids (AA) bioaccessibility and relative % of free essential and non-essential AA.

more importantly during the gastric phase than during the intestinal phase, since they were mainly positioned on the *sn*-3 position of the glycerol backbone, corresponding to the regioselectivity of the gastric lipase (Bourlieu et al., 2015). Notably, the fatty acid C18:1n9c constituted 42% of the lipid profiles in the intestinal phase and only 20% of the lipid profiles in the gastric phase for the formulas F2 and F3. For F1 the profiles were quite similar between the two phases, the fatty acid C18:1n9c constituted in both phases more than 53% of the fatty acid profiles. Difference in the pattern of FAs released in the gastric phase due to their position on the glycerol backbone (e position specially at the Sn-3) certainly leads to differences in the kinetics of FAs emptying and possibly on FAs absorption that might be different between the formulas containing only vegetable oil versus a mix of vegetable oil and bovine cream.

The differences in the fatty acid profiles resulted in different ω6/ω3 PUFA ratios between formulas (Fig. 1D). In the gastric phase, the ω6/ω3 PUFA ratios were similar between each other. In the intestinal phase, the formulas with bovine cream had a lower ω6/ω3 PUFA ratio than F1. The addition of bovine cream in F2 and F3 resulted in a lower ω6/ω3 PUFA ratio compared to F1 in the intestinal phase. A lower ω6/ω3 PUFA ratio may have important physiological effects, such as a decrease in the production of pro-inflammatory compounds generated from the arachidonic acid, resulting in improving inflammatory conditions and protection against oxidative stress (Di Pasquale, 2009). Furthermore, different studies on the elderly population showed that ω3 fatty acids were effective for treating elderly patients with depression, especially people with mild to moderate depression (Bae & Kim, 2018). In addition, ω3 can mitigate the ageing-related loss of muscle mass and strength (Candow et al., 2012) and protect from vascular diseases (Baierle et al., 2014).

3.3. Proteolysis

The DP was different between formulas during the 240 min of gastric digestion and reached 5.2% and 3.9% for F1 and F3 and up to 10.4% for the F2 (Fig. 2A). SDS-PAGE (Fig. 2B) showed the hydrolysis of the protein during the gastric digestion. After 45 min of digestion, no intact caseins (MW: 25–36 KDa) were visible on gels whatever the formulas. β-Lactoglobulin (MW: 19.9 KDa) and α-lactalbumin (MW: 16.2 KDa) were more resistant to the pepsin in the gastric digestion but at the end of the gastric phase, became undetectable. In line with other studies on milk protein digestion, caseins were digested more rapidly than the whey proteins in the gastric phase of our elderly *in vitro* digestion model. It is known that, *in vivo*, the low gastric pH induces casein coagulation and consequently decreases the gastric emptying time, while whey proteins remain soluble and are quickly emptied. The high protein content of the formulas together with a slower decrease in pH in the elderly digestive conditions than in the adult ones (pH of 3.7 at $T_{1/2}$ for the elderly vs 3.0 for the adult) resulted in an easier casein coagulum formation. These different emptying times, linked to the nature of the proteins, can change the intestinal kinetics of digestion and the whole-body postprandial amino acids metabolism (Hall et al., 2003). Although whey proteins allowed a faster release of free amino acids, higher hypo-aminoacidemia were observed after 4 h of whey protein ingestion in comparison to the ingestion of whole milk protein. A too-fast dietary amino acid delivery cannot support the anabolic requirement throughout the postprandial period (Lacroix et al., 2006). Due to this *in vivo* evidence, it is important to add casein in elderly formulas, in order to cover the anabolic postprandial period in the elderly population.

During intestinal digestion, the kinetics of the DP were different between the experimental formulas; the two formulas containing bovine cream (F2 and F3) had the same kinetics of digestion and up to 50.8 ± 2 and 52.9 ± 2.5 at the end of the intestinal step and were statistically different from that of F1 ($p < 0.01$) although this difference was<5%. that reached 56.8 ± 2 at the end of the digestion. In agreement with the intestinal DP, a similar behavior of the free AAs released was observed between F2 and F3 and statistically different ($p < 0.01$) even though the difference were<4% from that of F1 (Fig. 2C). The free AAs profile (Fig. 3) was composed of more than 50% essential AAs and the percentage as free essential AAs increased until 240 min of digestion in all samples. The AAs profiles were similar for the three experimental formulas. Lysine, arginine, leucine, tyrosine and phenylalanine were the major free AAs and for the majority of the AAs (60%), the kinetics of digestion was the same between F2 and F3. F2 and F3 had a slightly lower bioaccessibility of leucine than F1; Leu is an essential AA present in higher quantity in the whey proteins than in caseins (Horstman et al., 2021) that is essential to fight sarcopenia (loss in muscle mass during ageing). At the end of the intestinal phase, more than 80% of the total arginine and more than 50% of total leucine, phenylalanine, lysine and tyrosine were released (Supplementary data Figure 5).

3.4. Combined effect of lipolysis and proteolysis

PCA was performed in order to describe the combined effect of lipolysis and proteolysis and compare the formulas. In the PCA, for both the gastric and intestinal phases, more than 80% of the variance was explained by the two axes (85.4% and 88.3% for the gastric and intestinal phases respectively).

In the gastric phase (Fig. 4A), sample F1 was clearly separated from

the other formulae and were positively correlated to the first dimension. FFA profile and the DL had higher contributions to the first dimension, and F2 and F3 formulas were characterized by higher levels of saturated FFAs and a lower DL than F1. The second dimension can allow the separation of the two gastric time points, where the digesta at G180 and G240 were negatively and positively correlated to this dimension, respectively. The DP and the size of the particles were positively and negatively correlated to the second dimension respectively; these two parameters increased and decreased respectively during the gastric digestion. In the intestinal phase (Fig. 4B), formulas shared a similar DP and DL that increased throughout digestion together with the increase of the free essential AAs released. The F2 and F3 formulas were clearly separated from the F1 formula due to their negative correlation to the second dimension where they presented higher quantities of saturated fatty acids and lower AA bioaccessibility.

The native protein in formula F2 did not modify the kinetics of digestion of neither proteins nor lipids in comparison with the formula F1 and F3 that contain a milk protein isolate powder. The potential effect of the native protein on the digestion might have been affected by the final heat treatment that the formula received during its manufacture. The similarity in the kinetic of digestion between formulas was also confirmed by the similar evolution in the dimension of the particle size during gastric phase (Supplementary data Fig. 2). A strong evolution in the particle size was found after 45 min of digestion. The size of the particles decreased for all samples until reaching a size of 19 μm for the experimental samples. When the samples were analyzed with SDS (data not shown), the profiles of the samples had a similar particle size distribution as before digestion suggesting that only aggregation occurred in the gastric phase.

The addition of bovine cream seemed to slightly reduce the overall degree of proteolysis and the released of free AAs. The higher PLs concentration in the formula containing bovine cream (F2 and F3) compared to the formula with less PLs (F1), may explain the differences found in the degree of protein hydrolysis. Indeed, it has been already reported that PLs can interact with the digestion of the β-lactoglobulin (Mandalari et al., 2009). PLs have shown to bind the main calyx of the β-lactoglobulin, limiting protein digestion by trypsin and chymotrypsin. Indeed, the concentration of the free AAs in F2 and F3 was lower than for F1. Another possible explanation is that changing the nature of lipids of a formula may affect the interface of the lipid droplet. Proteins bound at the interface will see their structure demonstrated as it was stated by Maldonado-Valderrama et al. (Maldonado-Valderrama et al., 2008). Finally, the products of lipolysis (i.e. mono and diglycerides, lysophospholipids) have been shown to compete for access to the interface, and contribute to the dynamic changes occurring on the surface of the oil droplets, modifying protein digestion (Nik et al., 2010).

In both gastric and intestinal phases, the formulae with bovine milk were discriminated from the formula F1 due to their higher levels of saturated fatty acids. Recently, Nakamura et al. (2019) inversely correlated the consumption of total saturated fatty acids and the saturated fatty acids between C6:0 to C22:0 with the hypertension in a large cohort of elderly people (n = 240). In particular, C6:0 to C12:0 fatty acids were correlated with a decrease in the oxidative stress. Other studies showed a positive effect of long-chain fatty acids on hypertension; for instance, C18:0 fatty acids have been shown to significantly reduce blood pressure in elderly, limiting hypertension (Simon et al., 1996; Zheng et al., 1999). For these reasons, F2 and F3 formulas could be of special interest for elderly people.

3.5. Relevance of the DIDGI® system to investigate digestion in the elderly

In the present study and for the first time, the DIDGI® system was adapted to simulate the elderly digestive conditions. Indeed, the performance of the gastrointestinal tract is altered during ageing and differs comparing to the adult one. An exhaustive review of the scientific literature available has allowed to find how digestive parameters (digestive enzyme activities, bile salt concentration, pH of the oral cavity, stomach and small intestine, duration of the different phases etc) are affected by ageing. These parameters have been used to program the DIDGI® dynamic digestion simulator resulting in a fully dynamic gastrointestinal model of the elderly. Compared to static models, the DIDGI® system allows a fine regulation of the pH in the different compartments, the injection of digestive enzymes and bile in real time and the simulation of the food transit (gastric and intestinal emptying) in the gut. A recent review has shown that only three dynamic *in vitro* digestion models have been proposed so far (Lee et al., n.d.). The first studies were conducted by the group of Dr Uri Lesmes at Technion in Israel that investigated the *in vitro* digestion of pure proteins (Levi & Lesmes, 2014). Few years later, they used the model to compare the digestion of pure proteins and enteral nutrition formula in adult and elderly (Shani Levi et al., 2017). Another research group used the TIM (TNO gastro-Intestinal Model) system to study the *in vitro* digestion of cooked meat (Denis, Sayd, Georges, Chambon, Chalancon, Sante-Lhoutellier, et al., 2016). More recently, a chinese consortium used a biomechanical dynamic model to study the digestibility of apple polyphenolics (Shang et al., 2022).

When we look at the digestive parameters used in all these studies and the present one (enzyme activities, bile salt concentration etc), they are different showing that reaching a consensus at the international level, like it was done for the static model of adults within INFOGEST (Brodkorb et al., 2019), would be highly beneficial allowing to compare data obtained in identical conditions between studies. Such harmonization work is currently in progress within the INFOGEST network.

Finally, the main limitation of the DIDGI® system relies on the fact that there is no absorption in the small intestine and the digestion products remain in the gut lumen. The new generation of the DIDGI® system is now equipped with a hollow fiber connected to the small intestine and should allow to mimic passive absorption of nutrients. The next step will be to use this new device with the digestive parameters used in the present study in order to determine how nutrient removal upon time would affect our data.

4. Conclusion

In conclusion, the process used to generate the milk protein ingredient (liquid vs spray drying), did not lead to significant differences in the kinetics of lipid or protein digestion. The most important differences observed between formulas relied on the nature of the lipid source used (blend of vegetable oils for F1 vs vegetable oil + bovine cream for F2 and F3). As an example, C18:0 was significantly more released in F2 and F3 than in F1; it has to be noticed than this fatty acid, when reaching the bloodstream, decreases hypertension in the elderly (Zheng et al., 1999). Addition of bovine cream to the lipid fraction also led to a better ratio of ω6/ω3 in the FFAs released which is also beneficial for elderly people. For all these reasons, F2 and F3 appear more relevant for elderly people than F1. However, digestion of F1 ended up with higher amino acid bioaccessibility, especially Branched-Chain Amino Acids (Leucine, Isoleucine and Valine that are known to restore muscle protein synthesis (Rieu et al., 2007) and could be particularly adapted to elderly suffering from sarcopenia.

CRediT authorship contribution statement

Stefano Nebbia: Methodology, Formal analysis, Investigation, Writing – original draft, Data curation. **Olivia Ménard:** Methodology, Formal analysis, Investigation, Supervision, Writing – review & editing, Data curation. **Marie-Françoise Cochet:** Methodology, Formal analysis, Investigation. **Gwénaële Henry:** Methodology, Formal analysis, Investigation. **Nathalie Daniel:** Methodology, Formal analysis, Investigation. **Lorraine Moran:** Writing – review & editing, Data curation. **Kate Lennon:** Writing – review & editing, Data curation. **Grainne Dollard:** Writing – review & editing, Data curation. **Cian Moloney:** Writing –

review & editing, Supervision, Data curation. **Michelle Collins:** Writing – review & editing, Conceptualization, Project administration. **François Morgan:** Writing – review & editing, Conceptualization, Project administration. **Didier Dupont:** Writing – review & editing, Conceptualization, Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112716.

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