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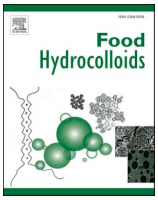

In vitro dynamic digestion of model infant formulae containing lactoferrin and medium chain triacylglycerols

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ABSTRACT

Four different model infant formulae were compared under simulated conditions of the infant gastrointestinal tract using an *in vitro* dynamic digestion protocol. These formulae were based on whey protein isolate, whey protein isolate + lactoferrin, long chain triacylglycerols or long + medium chain triacylglycerols. In each of the four cases, the influence of protein and lipid composition on their subsequent structural disintegration, proteolysis and lipolysis during digestion was investigated. Structural characterization of the four types of infant formulae was done before and during simulated digestion using confocal microscopy and laser light scattering methods. Proteolysis was followed using molecular weight distribution methodology, primary amine quantification, amino acid bioaccessibility and peptide analyses. Lipolysis was determined by gas chromatography. During gastric digestion, the structure of infant formulae based on lactoferrin was barely affected by the physicochemical conditions in the gastric chamber and all the protein fractions were found to be resistant to gastric proteolysis, except for α-lactalbumin. The degree of lipolysis within the gastric and intestinal phase was influenced by the lipid composition, noting that those formulae containing medium chain triacylglycerols exhibited a higher extent of lipolysis. These results are relevant for the development of formulae enriched with functional ingredients that may provide bioactive peptides and faster energy delivery to the newborn baby.

1. Introduction

Although human milk is preferred for infant nutrition, breastfeeding might not be possible and commercial infant formulae are used to meet the baby's nutritional needs (Martin, Ling, & Blackburn, 2016). These products are available in liquid and powder forms. They are produced from emulsions that have as the main components, milk proteins, phospholipids, carbohydrates and a blend of vegetable oils (Drapala, Auty, Mulvihill, & O'Mahony, 2017; Furtado, Carvalho, & Hubinger, 2021; McSweeney, Healy, & Mulvihill, 2008; McSweeney, Mulvihill, & O'Callaghan, 2004).

Whey proteins from bovine milk are widely used in infant formulae due to their nutritional and functional characteristics (McCarthy et al., 2012; McSweeney et al., 2008; McSweeney et al., 2004; Mulcahy, Park, Drake, Mulvihill, & O'Mahony, 2016). They represent 15–22% of all bovine milk proteins, with the major fractions being β-lactoglobulin, α-lactalbumin and serum albumin, in addition to other important minor proteins such as lactoferrin (Jensen, 1995). Lactoferrin is an 80 kDa glycoprotein, belonging to the group of transferrin. It has a high iron binding capacity that facilitates iron diffusion (Roseanu & Brock, 2006; Stowell, Rado, Funk, & Tweedie, 1991) and is thus very important for increasing the bioavailability of iron to the breastfed baby (Conesa et al., 2009). Furthermore, lactoferrin imparts antimicrobial attributes (Story & Perish, 2008) and can also help by improving bone health (Cornish & Naot, 2010) and immunomodulatory activity (Brock, 2002). Human lactoferrin and bovine lactoferrin, despite some biochemical and structural differences (69% sequence homology (Pierce et al., 1991)), have a similar bioactivity (Farnaud & Evans, 2003) and the use of bovine

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lactoferrin in infant formulae has been demonstrated as safe (Chierici, Sawatzki, Tamisari, Volpato, & Vigi, 1992).

Lipids are essential for infant nutrition and represent the major source of calories (Bourlieu et al., 2015; Lindquist & Hernell, 2010). Therefore, different blends of vegetable oils used in infant formulae seek to reproduce the lipid composition of human milk in order to achieve a better lipid absorption (Berger, Fleith, & Crozier, 2000). The mechanism of digestion and absorption for medium chain triacylglycerols (MCTs) is different to that for long chain triacylglycerols (LCTs) (Álvarez & Akoh, 2015). MCTs (containing 6–12 carbon atoms) move directly to the liver (through the hepatic portal) where they are oxidized to ketones, thus providing a rapid source of energy. For LCTs (containing more than 14 carbon atoms) absorption occurs through the intestinal lymphatic ducts where the lipids are carried in chylomicrons until reach the systemic circulation (Seaton, Welle, Warenko, & Campbell, 1986). In addition, MCTs have been reported to contain antimicrobial and antiviral properties (Álvarez & Akoh, 2015), and stimulate an increase in diet-induced thermogenesis and satiety (Papamandjaris, Macdougall, & Jones, 1998).

Although it is difficult to closely reproduce the physicochemical and physiological conditions of the human gastrointestinal digestion system, *in vitro* digestion protocols have been widely used (Bohn et al., 2018; Hur, Decker, & McClements, 2009). These protocols seek to reproduce the conditions in the mouth, stomach and small intestine to allow the study structural changes and release of food components (Parada & Aguilera, 2007). However, the simplest design of *in vitro* protocols (static) cannot mimic the dynamic flow of food and secretions. Consequently, a range of more elaborate dynamic digestion protocols have been created (Dupont et al., 2019; Guerra et al., 2012). Key features include pH regulation and transient flow conditions of both enzymes and foods. To simulate a baby's digestion, Ménard et al. (2014) built an apparatus that works under dynamic conditions to study the changes occurring in the food during digestion. The related parameters were adjusted and based on a review of collected digestion data measured for babies *in vivo* (Bourlieu et al., 2014): subsequent results showed the simulation closely followed the *in vivo* digestion data (Ménard et al., 2014). Since then, this apparatus has been successfully used to study the *in vitro* dynamic digestion of human milk and infant formulae (De Oliveira, Deglaire, et al., 2016; Deglaire et al., 2016; Le Roux et al., 2020; Nebbia et al., 2020) allowing the identification of the influence of food structures on the performance of the baby's digestion process.

In this context, there is a frequent need to develop new dietary formulations that contain functional appeal to meet the scientific, nutritional and technological demands. The characterization of infant foods in a simulated gastrointestinal tract working under dynamic conditions is of utmost importance to establish whether the use of proteins or lipid sources will influence the proteolysis and lipid digestion, and thus provide relevant information for possible technological applications in this food area. Nonetheless, there appear to be few previous studies on the *in vitro* dynamic digestion of model infant formulae that are composed of two or more functional ingredients, such as lactoferrin and medium chain triacyclglycerols. The purpose of this study was to evaluate the digestion of four different model infant formulae under simulated conditions of the infant gastrointestinal tract using an *in vitro* dynamic digestion protocol. These products were based on either whey protein isolate or whey protein isolate + lactoferrin (50:50) and on long chain triacylglycerols or long + medium chain triacylglycerols (75:25).

2. Material and methods

2.1. Material

Whey protein isolate from bovine origin (WPI) (Arla Foods Ltd., Denmark), lactoferrin from bovine origin (LF) (Synlait Milk Ltd., New Zealand), lactose (LAC) (Alibra Ingredientes Ltda., Brazil), maltodextrin 10 DE (MALTO) (Ingredion Ingredientes Industriais Ltda., Brazil), high oleic sunflower oil (HOSO, rich in long chain triacylglycerols) (Bunge Alimentos S.A., Brazil) and a mixture of caprylic and capric triglycerides (medium chain triacylglycerols, MCT) (Stepan Company, USA) were used to produce the model infant formulae. For the digestion trials, rabbit gastric extract (RGE) was obtained from Lipolytech (France). Porcine pancreatin (PAN) (P7545), bovine bile extract (B8631), and the enzyme inhibitors pefabloc (76,307), pepstatin A (P5318) and 4-Bromophenylboronic acid (B75956) were obtained from Sigma-Aldrich (France). All other reagents were of analytical grade. Enzyme activities were determined as described by Brodkorb et al. (2019).

2.2. Methods

2.2.1. Model infant formulae (IFs)

Table 1 sets out the composition of the four model infant formulae prepared by following procedures set out by Furtado et al. (2021). All formulations respected the concentration limits of carbohydrates, protein and oil as established by the specific Brazilian regulation of infant formulas (Brazil, 2011). The resulting powders were rehydrated according to instructions for preparing commercial infant formulas. They were dispersed in preheated water (70 °C) in a baby bottle. The final protein and oil concentration of the feeds were 2.03 ± 0.04 and 3.00 ± 0.12%, respectively.

2.2.2. Conditions used in the in vitro dynamic digestion chamber

Gastrointestinal digestion trials were performed in triplicate for each milk formulation in an *in vitro* dynamic system known as DIDGI® (Ménard et al., 2014). The apparatus consists of two compartments that mimic the stomach and the small intestine: it is controlled by a software package known as STORM®. The gastric and intestinal parameters used were based on those observed for the digestion of human milk (De Oliveira, Bourlieu, et al., 2016) except that the gastric and intestinal emptying were adapted for infant formulae. The specific conditions for digestion are given in Table 2. The Elashoff equation (Elashoff, Reedy, & Meyer, 1982) (Eq. (1)) was used to calculate the gastric and intestinal emptying. F represents the remaining chyme fraction in the stomach, t the delivery time, $t_{½}$ the half delivery time and β describes the shape of the curve. Values for these parameters were based on data from previous studies (Ewer, Durbin, Morgan, & Booth, 1994; Ménard et al., 2014).

$$F = 2^{-\left(\frac{t}{t_{1/2}}\right)^{\beta}} \quad (1)$$

Each digestion experiment lasted 3 h, with aliquots taken before digestion (time 0 min, G0) and during digestion in the gastric (G) and intestinal (I) compartments at 40, 80, 120 and 180 min. Structural characterization of the samples was performed immediately. Those aliquots taken for proteolysis analysis were frozen at −20 °C, after the addition of a protease inhibitor (10 μL of 0.72 mM Pepstatin A per mL of gastric digesta or 50 μL of 0.1 M Pefabloc per mL of intestinal digesta). Lipase inhibitor was added to aliquots for lipid analysis (50 μL of 0.1 M 4-Bromophenylboronic acid per mL of digesta) which were immediately submitted for lipid extraction.

2.2.3. Structural characterization

2.2.3.1. Particle size measurement. Particle size distribution was carried

Table 1
Composition of the rehydrated IF powders.

Formulation	Aqueous Phase (%, w/w)					Oil Phase (%, w/w)	
	Water	WPI	LF	LAC	MALTO	HOSO	MCT
F1	88	2	0	3	4	3	0
F2	88	1	1	3	4	3	0
F3	88	2	0	3	4	2.25	0.75
F4	88	1	1	3	4	2.25	0.75

WPI - whey protein isolate, LF – lactoferrin, LAC – lactose, MALTO – maltodextrin, HOSO - high oleic sunflower oil, MCT - caprylic/capric triglyceride

Table 2
Conditions recorded for gastric and intestinal digestion trials.

Gastric Conditions	
Simulated Gastric Fluid (SGF) (pH 6.5)	Na^+: 94 mmol/L K^+: 13.2 mmol/L Cl^-: 122 mmol/L
Temperature	37 °C
IF feeding	10 mL/min from 0 to 10 min
Acidification curve (adjusted with HCl 0.5 M)	pH = − 0.0155*time (min) + pH_{IF}
RGE (diluted in SGF)	1 mL/min from 0 to 10 min
(268 U pepsin/mL and 19 U lipase/mL)	0.5 mL/min from 10 to 180 min
Gastric emptying dynamics	$t_{1/2}$ = 78 min, β = 1.2
Intestinal Conditions	
Simulated Intestinal Fluid (SIF) (pH 6.6)	Na^+: 164 mmol/L K^+: 10 mmol/L Ca^{2+}: 3 mmol/L
Temperature	37 °C
pH	6.6
Bovine bile extract (diluted in SIF) (3.1 mmol/L)	0.5 mL/min from 0 to 180 min
Porcine pancreatin (diluted in SIF) (90 U lipase/mL)	0.25 mL/min from 0 to 180 min
Intestinal emptying dynamics	$t_{1/2}$ = 200 min, β = 2.2

out using a Multi-Angle Static Light-Scattering Mastersizer 2000 (Malvern Instruments, UK) set up with 2 lasers sources (633 and 466 nm). Distilled water was used as the dispersant medium. The refractive indexes used were 1.462 and 1.333 for vegetable oil and for water, respectively. The mean particle diameter (in μm) was expressed as the volume-weighted average ($D_{4,3}$) (Eq. (2)).

$$D_{4,3} = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \quad (2)$$

where n_i is the number of droplets and D_i. is the droplet diameter.

2.2.3.2. Confocal laser scanning microscopy (CLSM). A Confocal Zeiss LSM880 microscope (Zeiss, Germany) with a 63× magnification objective lens was used to observe the IFs before and during digestion. Samples were stained with four fluorescent dyes (Nile Red (in the gastric samples) or Lipidtox™ for apolar lipids (in the intestinal samples), Rhodamine-PE for amphiphilic compounds (in the intestinal samples), and Fast Green FCF for proteins (in both gastric and intestinal samples)). The ZEN Lite black software (Zeiss, Germany) was used to process the images produced.

2.2.4. Proteolysis

The extent of proteolysis was investigated by electrophoretic analyzes (SDS-PAGE), primary amine quantification, amino acid bioaccessibility and peptide analyses, as described below.

2.2.4.1. SDS-PAGE. The molecular weight distribution of the proteins in samples was evaluated by SDS–PAGE under reducing conditions. 4–12% polyacrylamide NuPAGE® Novex® Bis-Tris 15 well precast gels (Invitrogen, USA) were used according to De Oliveira, Bourlieu, et al. (2016). Samples were diluted with NuPAGE® LDS sample buffer and distilled water. Reducing conditions were induced by the addition of 0.5 M DL-dithiothreitol to reach a final concentration of 0.05 M. A molecular weight marker (Invitrogen), RGE and porcine pancreatin were used as the controls. Aliquots of 20 μL sample (containing 4 μg of protein) were loaded and the migration was performed at 200 V and 50 mA/gel. Gels were fixed by the addition of the fixing solution (ethanol (30 vol %), acetic acid (10 vol %) and deionized water (60 vol %)). They were then rinsed for 15 min with deionized water before staining with Coomassie Blue with further de-staining by repeated washings with water. Images of the gels were taken and processed using Image Scanner III software (GE Healthcare Europe GbmH, France).

2.2.4.2. Primary amine quantification. The methodology set out by Nielsen, Petersen, and Dambmann (2001) based on *o*-phthaldialdehyde was used to quantify the primary amines in the samples (soluble fraction), following centrifugation (10,000·xg at 4 °C for 20 min). A Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the absorbance (340 nm) using methionine (0–2 mM) as the standard for the calibration curve.

2.2.4.3. Amino acid bioaccessibility. Total amino acids in rehydrated IFs were determined following procedures set out by Davies and Thomas (1973) once samples had been subjected to acid hydrolysis (110 °C for 24 h). Free amino acids were measured following deproteinization of the samples by sulfosalicylic acid (0.5 g/mL) (Mondino, Bongiovanni, Fumero, & Rossi, 1972). Cation exchange chromatography was used to determine the amino acid composition: the apparatus was a Biochrom-30+ Amino Acid Analyzer (Biochrom Ltd., UK). Elution of samples was done with lithium citrate buffer, and the post-column derivatization was done with ninhydrin (Moore, Spackman, & Stein, 1958). Absorbance was measured at 570 nm (with the exception of 440 nm for proline) and amino acid quantification was based on the external calibration curve using standards for amino acids. The amino acid bioaccessibility (%) was evaluated from the relation between the amount of free amino acids (FAA, g/100 g of meal) released after 180 min of digestion and the amount of total amino acids (TAA, g/100 g of meal) present in the initial sample.

2.2.4.4. Peptide analyses. Mass spectrometry (MS) analysis of the samples was performed using a nano-RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, USA) and coupled to a nanoelectrospray ion source. The full procedure is given by Deglaire et al. (2016). Samples (taken before and after digestion) were diluted (200x) with buffer prior to injection. The μ-precolumn used to concentrate the samples (5 μL) was a pepMap100 (C18 column, 300 μm i. d. × 5 mm length, 5 μm particle size, 100 Å pore size; Dionex, The Netherlands). Separation was done with a PepMap RSLC column (C18 column, 75 μm i. d. × 250 mm length, 3 μm particle size, 100 Å pore size; Dionex). For each MS scan, the ten most intense ions were selected for MS/MS fragmentation and excluded from fragmentation for 15 s. X! TandemPipeline software (Langella et al., 2017) and a database composed of 6905 reviewed proteins of Bos Taurus (https://www.uniprot.org downloaded in January 2020) were used together to identify the peptides revealed by the MS/MS spectra. The search parameters on the database were (1), a non-specific enzyme cleavage selection, (2), a 0.01 Da mass error for fragment ions and (3), 10 ppm mass error for parent ions. In addition, oxidation of methionine and phosphorylation of threonine and serine were also selected as variables. Automatic validation of any peptides identified was done for those molecules with e-values < 0.01.

Each identified peptide was quantified using the MassChroQ software (Valot, Langella, Nano, & Zivy, 2011). An eXtracted Ion Chromatogram (XIC) was obtained by plotting the intensity of the signal for each m/z value for the peptide as a function of retention time: the area under the curve of the resulting signal was then used for statistical analysis. When a peptide with several charge states was found, all ion intensities were summed.

2.2.5. Lipolysis

The extent of lipolysis (%) (Eq. (3)) was calculated as the proportion of the free fatty acids (FFA, g/100 g of meal) released during digestion relative to the total fatty acids (TFA, g/100 g of meal) in the IFs (Carriere, Barrowman, Verger, & René, 1993).

$$Degree\ of\ lipolysis\ (\%) = \frac{100 * FFA}{TFA} \quad (3)$$

The total and free fatty acid composition was quantified using a gas chromatograph mass spectrometer. The model was a Shimadzu GCMS-

QP2010 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). 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 measurement 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 plate 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. Fatty acid identification

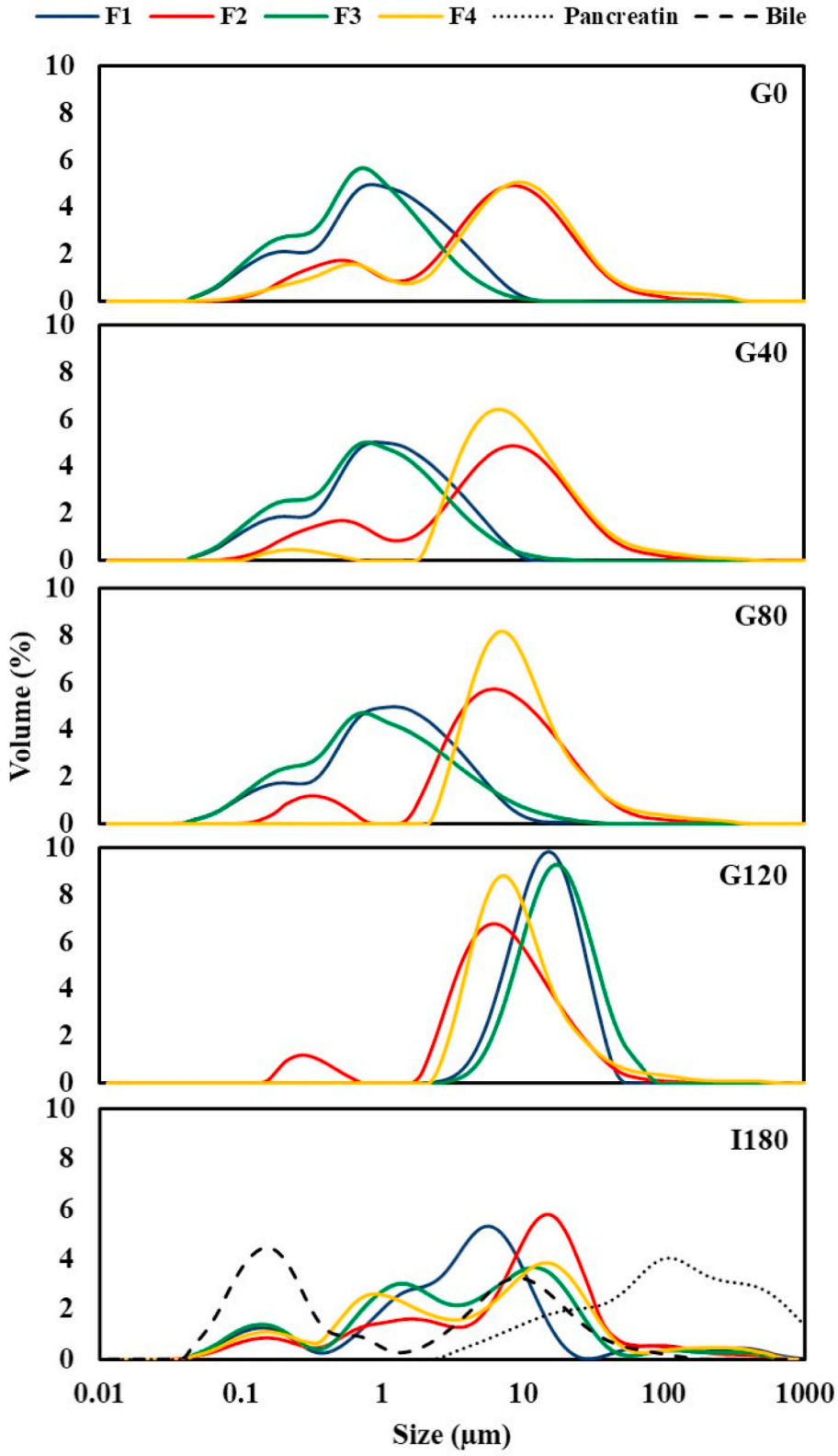

Fig. 1. Particle size distribution of the four IFs (G0) and of the resulting digesta at different times of the gastric (G40, G80, G120) and intestinal (I180) digestion phases.

was cross-checked by mass spectrometry using the NIST mass spectral database library (2017; www.nist.gov). GCMS solution software (Shimadzu Corp., Japan) was used for data acquisition. The qualitative and quantitative composition of the molecules found was identified/calculated using standards for fatty acids. Those samples intended for TFA determination were firstly submitted (along with an internal C13 standard) for transmethylation as described by López-López, López-Sabater, Campoy-Folgoso, Rivero-Urgell, and Castellote-Bargalló (2002). With respect to FFA determination, after collecting digesta samples, three internal standards (160 μL of C5, C11 and C19 at 0.5 mg/mL) were added to the samples and Folch extractions were done following the procedure given by Bourlieu et al. (2015). The phase rich in chloroform was collected and subjected to a solid phase extraction using the Strata® NH_2 SPE (1mL/100 mg) (Phenomenex, USA) in order to recover the FFA fraction which was subsequently methylated. Finally, samples were stored at - 20 °C until analysis by GCMS.

2.3. Statistical analysis

All digestion experiments were carried out in triplicate. The results are presented as the average with the standard deviation. Data was analyzed using analysis of variance (ANOVA) with significant differences ($p < 0.05$) being evaluated by the Tukey test. This procedure was done using the software Minitab (version 16.1.0). MS analysis results were statistically evaluated using a V-test ($p < 0.05$) and the Wilcoxon Rank Sum Test using R software (version 3.1.1). A heatmap was generated based on the log10-transformed values of peptide abundance; identification of clusters was done by applying the Ward method within the R software, this based on the Euclidian calculated distance between peptides.

3. Results and discussion

3.1. Structural characterization during digestion

The microstructure of the IFs was evaluated both before and during the gastrointestinal digestion process (Figs. 1–3). Measurements of particle size distribution were done before digestion and after 40, 80 and 120 min of gastric digestion and also at the end of the intestinal digestion (180 min). Confocal microscopy was done before digestion, following 120 min of gastric digestion and at the end of the intestinal digestion phase (180 min).

Before digestion (G0), all the IFs exhibited a bimodal particle size distribution (Fig. 1) with mean particle diameters ($D_{4,3}$) varying between 1.1 to 1.4 and 9.3–10.2 μm (Fig. 2) for IFs containing only WPI (F1 and F3) and WPI + LF (F2 and F4), respectively. The higher mean particle diameter for IFs containing LF (F2 and F4) is attributed to an extensive aggregation of protein/droplets noting that a pronounced electrostatic interaction between oppositely charged proteins, such as WPI and LF, may have occurred (Furtado et al., 2021; Li et al., 2018). This interpretation is corroborated by the microscopy images (Fig. 3), where those IFs containing WPI (F1 and F3) displayed oil droplets that were more uniformly distributed whereas IFs containing WPI + LF showed some protein and/or droplet aggregates.

In the gastric phase, the IFs remained structurally stable during the first 80 min since all the digesta samples presented bimodal particle size distribution as observed before digestion. After 120 min, there was an increase in the particle size of the IFs containing WPI (F1 and F3) (Figs. 1 and 2), suggesting an aggregation of the particles/droplets, which was also observed with confocal images (Fig. 3). However, the change in particle size was not significant for those IFs containing WPI + LF (F2 and F4) and the type of oil (whether HOSO or HOSO:MCT) did not affect the structural characteristics of the IFs. As previously described, emulsions stabilized by proteins are very susceptible to aggregation under gastric conditions since these proteins are hydrolyzed (Bourlieu et al., 2015; Singh, 2011; Singh, Ye, & Horne, 2009). At 120 min of gastric

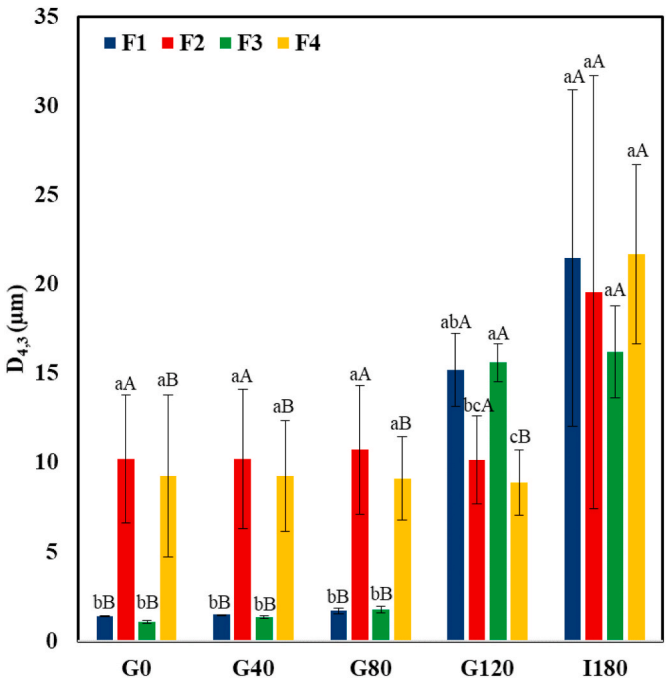

Fig. 2. Particle size ($D_{4,3}$) of the four IFs (G0) and of the subsequent *digesta* at different times of the gastric (G40, G80, G120) and intestinal (I180) digestion phases. Different lowercase letters between the four IFs at the same sampling time or different uppercase letters for the same sample as time progresses indicate significant difference ($p \leq 0.05$) was revealed by the Tukey test.

digestion, the pH (4.9–5.0) was close to the isoelectric point of WPI (5.2) (Ju & Kilara, 1998), and this might reduce the repulsive forces between particles/droplets leading to their aggregation (McClements, 2015). On the other hand, LF has a much higher isoelectric point (>8.0) (Steijns & van Hooijdonk, 2007), which could explain the smaller change in particle size during gastric digestion. The gastric emptying parameters applied in this study were the same for all four IFs, however some *in vivo* research has reported that structural differences in the feed may affect the rate of gastric emptying with possible further consequences on the absorption of the nutrients contained (Cavkll, 1981; Henderson, Hamosh, Armand, Mehta, & Hamosh, 2001; Marciani et al., 2007).

By the end of the intestinal digestion phase (I180) all of the aggregates originally present had disappeared, suggesting the total or partial hydrolysis of protein and lipid droplets in the feed. The resulting products of digestion (such as peptides and fatty acids) might be expected to be amphiphilic compounds, as shown in the confocal images (Fig. 3). The droplet/particle size distribution of the *digesta* became multimodal (Fig. 1), with a corresponding increase in the mean droplet/particle diameter ($D_{4,3}$) (Fig. 2). These larger particles could have been non-digested matter and/or coalesced lipid droplets, micelles or other particles from pancreatin and bile dispersion (as implied by the particle size distribution of pancreatin and bile dispersion (Fig. 1)).

3.2. Proteolysis

Protein hydrolysis during the *in vitro* dynamic gastrointestinal digestion trials was monitored by use of SDS-PAGE methodology (Fig. 4). Samples were taken on six occasions raging from 40 to 180 min of digestion (G40, G80, G120, G180, I40 and I180). These were analyzed and compared to the undigested IFs (G0). Electrophoretic profiles of the IFs (G0) highlighted bands near 14.4 and 21.5 kDa that relate to the major fractions in WPI: α-lactalbumin and β-lactoglobulin, respectively (Ménard et al., 2014). For IFs F2 and F4, a band between 66.3 and 97.4 kDa was also observed and relates to lactoferrin (80 kDa) (Lönnerdal & Iyer, 1995). Lactoferrin and β-lactoglobulin were resistant to proteolysis

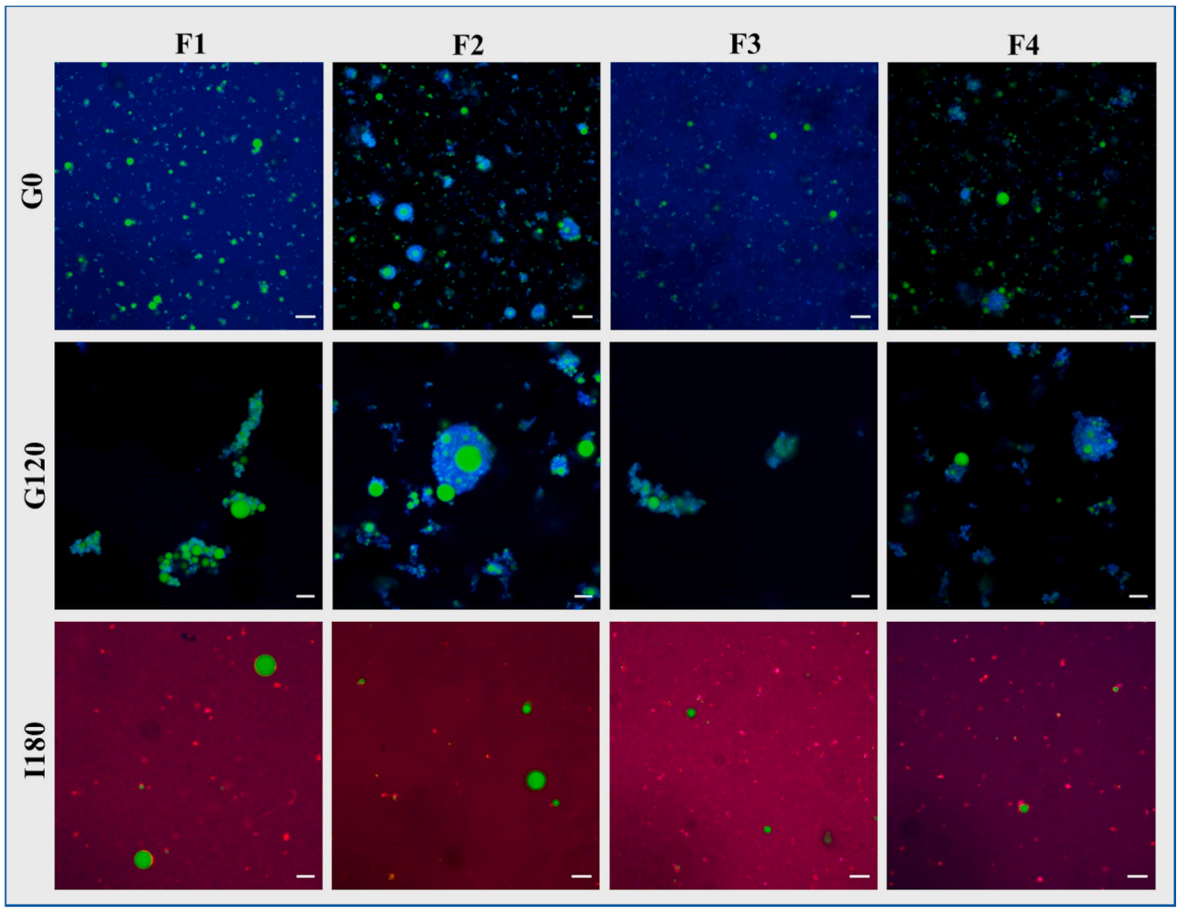

Fig. 3. Confocal laser scanning microscopy images of the four IFs (G0) and subsequent *digesta* at two points in the digestion process (G120 and I180). Proteins are stained in blue (Fast Green), lipids in green (Nile Red for G120 or Lipidtox for I180), and amphiphilic compounds in red (Rhodamine-PE (I180)). Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

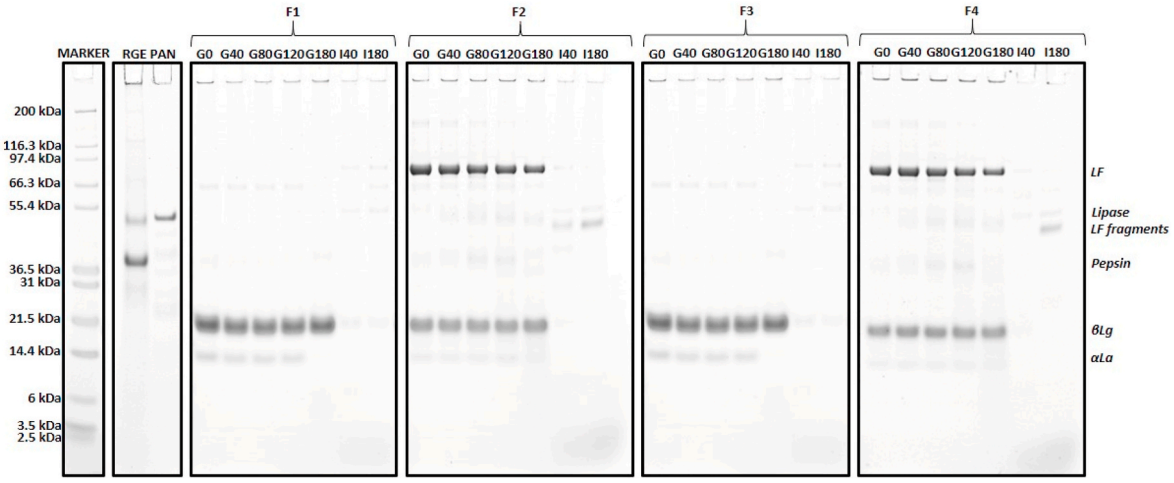

Fig. 4. SDS-PAGE electrophoretic profiles of the IFs (G0) and subsequent *digesta* at different times of gastric (G40, G80, G120, G180) and intestinal (I40 and I180) digestion.

during the gastric phase of digestion, however α-lactalbumin was almost fully hydrolyzed at the end of this process (G180) with the relating band being hardly visible. These results are in agreement with previous works (Bouzerzour et al., 2012; Bourlieu et al., 2015; Halabi, Croguennec, Bouhallab, Dupont, & Deglaire, 2020; Ménard et al., 2014). During the intestinal digestion, all the remaining proteins were rapidly hydrolyzed noting that the presence of their characteristic bands had disappeared by the end of this phase. Protein hydrolysis produced small peptides (below 6 kDa) and such intense protein hydrolysis during the intestinal phase is reported to occur due to the pH neutralization and the high pancreatic protease activity (Ménard et al., 2014; Singh & Sarkar, 2011). For gastric conditions, the samples exhibited a weak band near 36.5 kDa that relates to pepsin (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009) and another weak band near 55.4 kDa (mainly observed for samples during

the intestinal phase), which relates to lipase (Bakala N'Goma, Amara, Dridi, Jannin, & Carrière, 2012). For *digesta* from IFs F2 and F4, a band near that for lipase was also observed and relates to some LF fragments that were not fully hydrolyzed, as previously reported (Bokkhim, Bansal, Grøndahl, & Bhandari, 2016; Britton & Koldovsky, 1989; Halabi et al., 2020; Rastogi et al., 2014). These fragments may enable beneficial physiological effects such as an iron binding ability and antimicrobial properties (Rastogi et al., 2014; Steijns, 2001; Wakabayashi, Yamauchi, & Takase, 2006). Furthermore, such resistance to proteolysis is reported to be due to the presence of iron in native LF resulting in a compact molecular structure (Sánchez, Calvo, & Brock, 1992). No clear difference, in terms of proteolysis, was observed for IFs with the same protein composition, but formulated with a different lipid composition.

The concentration of primary amines in samples taken during digestion corroborates the findings from the SDS-PAGE analysis since no significant increase in primary amines was observed during gastric digestion (Fig. 5). This observation may be associated with the greater resistance to digestion of the globular whey proteins and the gastric conditions that had been adjusted to mimic the infant stomach. The relatively high gastric pH (4–6.8) and the low pepsin concentration resulted in a low activity of this enzyme, since its optimal pH is closer to 2 (Piper & Fenton, 1965). During the intestinal digestion phase, the concentration of primary amines greatly increased (≈4x) over the first 40 min and then, at a slower rate, up to180 min. Undigested IFs containing only WPI (F1 and F3), independently of their lipid composition, were slightly higher in primary amine content. However, after the intestinal digestion phase only IF F1 (containing HOSO) led to a *digesta* with a significantly higher content of primary amines, suggesting a lower interaction with the oil phase. These findings are supported by the results of amino acid bioaccessibility (Fig. 6), where the IF F1 led to a higher percentage of hydrolysis of methionine, tyrosine and lysine. The digestion of all the four IFs led to an amino acid bioaccessibility of more than 60% for tyrosine, phenylalanine and arginine, with most of these measurements falling in the same range of values of control IFs reported by Le Roux et al. (2020). However, a decreased bioaccessibility was noted for most of the amino acids of IFs containing medium chain triacylglycerols (HOSO:MCT) (F3 and F4) which may suggest a stronger interaction with the oil phase.

The results of the MS analysis conducted on the IFs before their digestion revealed between 218 and 231 peptides sequenced with a median molecular weight varying between 2352 and 2411 Da (Fig. 7). These peptides were most likely released by the endogenous proteases present in the bovine milk such as elastase or plasmin (Kelly, O'Flaherty, & Fox, 2006). Furthermore, although no casein was added to these IFs, casein peptides were still found and may be result of the cleavage ability of plasmin, making them soluble in whey fractions (Ismail & Nielsen, 2010). After digestion, the number of different peptides slightly increased for all samples, and the corresponding median molecular weight significantly falling ($p < 0.05$) to values between 1384 and 1417 Da (with no significant difference between the different IFs, $p > 0.05$). The number of peptides present after digestion is likely to be underestimated since the MS analysis only identifies those with between 6 and 51 amino acid residues. Thus, smaller peptide molecules were not included in the present dataset.

Most of the peptides identified in the four IFs were from β-lg (15%) and β-cas (27%). Some are known to induce biological activity, such as ACE-Inhibitory (LVYPFPGPIPN from β-cas) (Hernández-Ledesma, del Mar Contreras, & Recio, 2011) or antimicrobial properties (TPEVDDELEK from β-lg and PVVVPPFLQPE from β-cas (S. D. Nielsen, Beverly, Qu, & Dallas, 2017)). Furthermore, IFs containing WPI + LF (F2 and F4) resulted with peptides with antimicrobial properties (ENLPEKADRDQYE, FENLPEKAD, GSPPGQRDLLFKDSALGFLRIPS, RSVDGKEDL, SFQLFGSPPGQR, TLDGGMVFEAGRDPYKLRPV, TVFENLPEKA and VFEAGRDPY), all of them derived from the LF (Bai et al., 2010), as previously reported by Kuwata, Yip, Tomita, and Hutchens (1998). Fig. 8 presents a heatmap containing the 412 peptides identified for all samples. Based on similarity, they were grouped into 7 clusters and the distribution of the peptides was detected within each cluster. A large part of the peptides identified in all IFs before digestion belongs to cluster 4 ($n = 160$), where 74% of the peptides included originated from α- and β-casein peptides. These peptides were characterized by a significantly higher length and molecular weight ($p < 0.05$), as demonstrated by results presented in the boxplot (Fig. 7). Peptides grouped under cluster 4 were totally digested after 180 min of digestion. Peptides from β-lg were also found in the IFs, to a lesser extent before and after digestion (cluster 6) and to a greater extent only after digestion (clusters 5 and 6). 39, 31 and 16% of the β-lg peptides were found in clusters 1, 5 and 6, respectively. Clusters 1, 5 and 7 represent peptides released only after digestion, where clusters 1 and 5 are mostly represented by β-lg, k- and β-casein peptides (59 and 76%, respectively), whereas cluster 7 mostly comprises the LF peptides (60% of LF peptides were present in this cluster) from IFs F2 and F4.

3.3. Lipolysis

Lipid digestion of the IFs (Fig. 9) is presented in terms of the degree of lipolysis. During digestion, the rate of lipolysis might be regulated by the mobility of lipases to interact with the dispersed oil phase (Golding et al., 2011). Despite the nature of *digesta* (aggregated particles), which is a limiting factor for the degree of lipolysis (Nguyen, Bhandari, Cichero, & Prakash, 2018), there were clear differences in lipolysis between the four IFs after 120 min of gastric digestion. Those IFs containing only WPI (F1 and F3) resulted in a higher degree of lipolysis during gastric digestion (10.6 ± 1.2 and 40.3 ± 6.8, respectively) than those containing LF (2.2 ± 0.6 and 22.2 ± 6.0, respectively). This finding corresponds to the results of primary amines, suggesting a lower interaction of the proteins with the oil phase, thus facilitating lipase action. However, during intestinal digestion, the degree of lipolysis was not

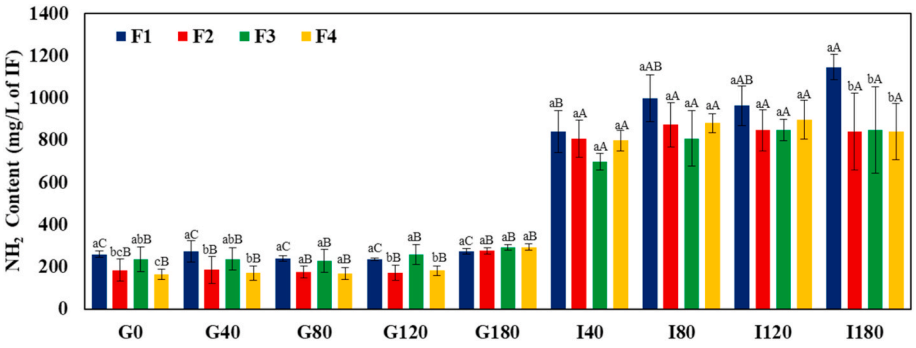

Fig. 5. Concentration of primary amines (NH_2) in the soluble fraction of the four IFs (G0) and in the subsequent *digesta* at different times of gastric (G40, G80, G120, G180) and intestinal (I40, I80, I120, I180) digestion. Different lowercase letters between the four IFs at the same sampling time or different uppercase letters for the same sample as time progresses indicate significant difference ($p \leq 0.05$) was revealed by the Tukey test.

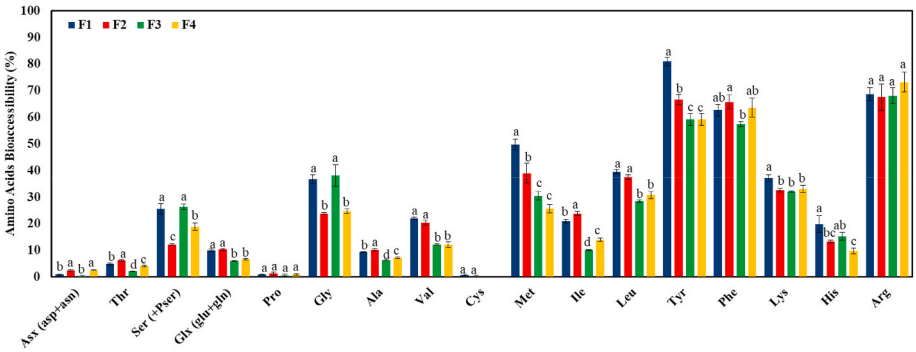

Fig. 6. Bioaccessibility of the amino acids derived from the digestion of the four IFs after the gastric and intestinal phases. Different letters amongst samples for the same amino acid indicate a significant difference ($p \leq 0.05$) with respect to the Tukey test.

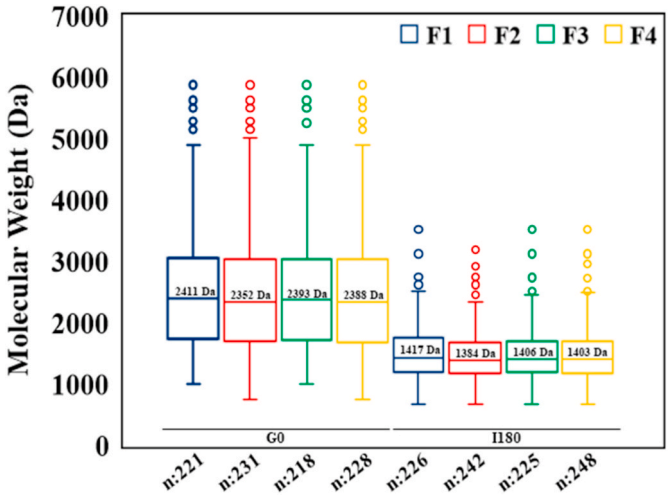

Fig. 7. Median molecular weight and total number of peptides identified for IFs samples before (G0) and after digestion (I180).

significantly influenced by the protein composition: possibly due to extensive protein hydrolysis. Even so, previous studies have reported that bile salts can interact (electrostatically) with lactoferrin, thus hindering the interaction of lipase with the lipids (Sarkar, Horne, & Singh, 2010; Zhang, Zhang, Zhang, Decker, & McClements, 2015). On the other hand, the degree of lipolysis was strongly influenced by the composition of lipids present. Those IFs containing medium chain triacylglycerols (F3 and F4) presented significantly higher lipolysis (67.4 ± 4.4 and 77.0 ± 11.0, respectively) than those containing only long chain triacylglycerols (F1 and F2, 44.0 ± 9.7 and 33.8 ± 12.5, respectively). This may be due to the carbon chain length of the associated fatty acids, since those with a shorter chain have higher water affinity, thus facilitating their migration into the aqueous medium thus avoiding the inhibition of lipase activity at the interface (Li & McClements, 2010).

The molecular weight of medium chain triacylglycerols is smaller than that of long chain triacylglycerols, thus enabling greater pancreatic lipase activity (Bach & Babayan, 1982). The long chain FFAs are also prone to concentrate at the oil–water interface and thus tend to inhibit lipase activity by the mechanism of displacing the lipase molecules from this active zone (Pafumi et al., 2002; Sek, Porter, Kaukonen, & Charman, 2002). In this way, the hydrolysis of medium chain triacylglycerols occurred in a faster and more effective way than long chain triacylglycerols. IFs containing medium chain triacylglycerols led to a higher degree of lipolysis than raw human milk after dynamic digestion (around 62%) (De Oliveira, Deglaire, et al., 2016). However, the current study was carried out with model infant formulae which need to be improved to meet all nutritional requirements, if the benefits previously mentioned are to be achieved. Furthermore, although MCTs led to faster absorption than LCTs, further absorption studies are still needed (Wei, Jin, & Wang, 2019).

4. Conclusion

The nature of the ingredients used in preparing infant formulae (especially protein and lipid composition) clearly affected their digestion. In terms of IFs microstructure, particle aggregation (lipid droplets and proteins) was observed during the gastric phase for formulae based on WPI: this was attributed to the physicochemical conditions of the gastric medium. On the other hand, the formulae based on LF were less affected by the gastric conditions. All the protein fractions present in the four IFs showed to be resistant to proteolysis during the gastric phase (except for α-lactalbumin), with subsequent extensive protein hydrolysis in the intestinal digestion phase. The different lipid composition in the four IFs did not affect the protein hydrolysis and likewise, the protein composition of the IFs did not affect the lipid digestion. However, the lipid composition influenced the ability of lipase to adsorb at the oil–water interface and consequently the extent of lipid digestion. The degree of lipolysis within the gastric and intestinal phase was significantly higher for those IFs containing medium chain triacylglycerols; an observation which could be attributed to their higher affinity for water. These findings are relevant for the design of enriched LF and MCT based infant formulae that could provide functional proteins and faster energy to newborn babies. Nevertheless, the bioavailability of these functional ingredients needs to be assessed by *in vivo* studies to more reliably mimic the gastrointestinal conditions of infants.

Credit authorship contribution statement

Guilherme F. Furtado: Conceptualization, Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. Olivia Ménard: Formal analysis, Methodology, Writing – review & editing. Xiaoxi Yu: Methodology, Writing – review & editing. Jordane Ossemond: Formal analysis, Methodology, Writing – review & editing. Gwénaële Henry: Formal analysis, Methodology, Validation, Writing – review & editing. Valérie Briard-Bion: Formal analysis, Methodology, Validation, Writing – review & editing. Julien Jardin: Formal analysis, Methodology, Validation, Writing – review & editing. Amélie Deglaire: Supervision, Writing – review & editing. Miriam D. Hubinger: Supervision, Writing – review & editing, Project administration, Funding acquisition. Didier Dupont: 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

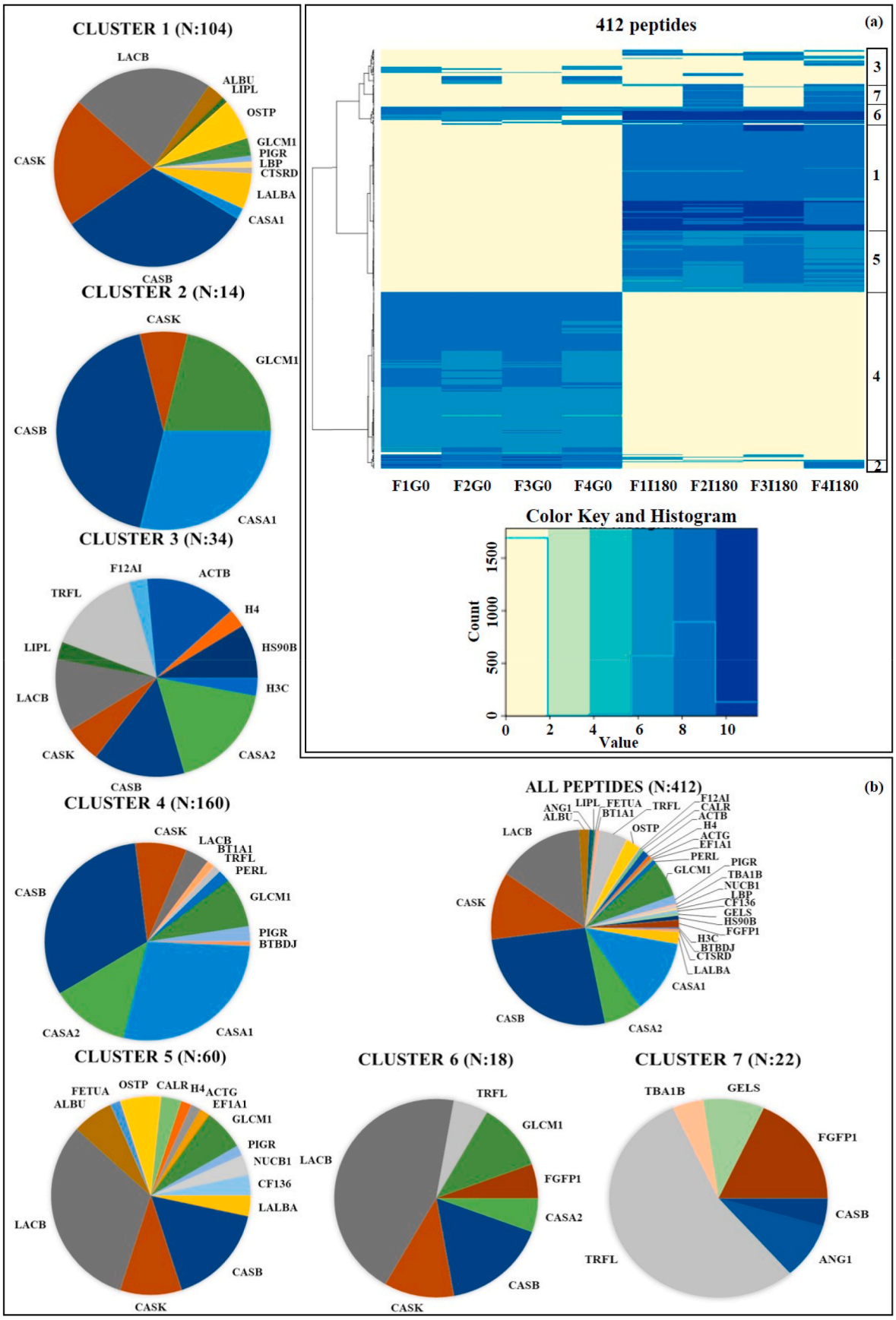

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Fig. 8. (a) Heatmap of log10 transformed abundances for all peptides (n = 412) identified by NanoLC–MS/MS for IFs before (G0) and after their digestion (I180) and (b) the distribution of the peptides detected within each cluster. Abbreviations: H3C: Histone H3.3C, BTBDJ: BTB/POZ domain-containing protein, CTSRD: Cation channel sperm-associated protein subunit, LALBA: Alpha-lactalbumin, CASA1: Alpha-S1-casein, CASA2: Alpha-S2-casein, CASB: Beta-casein, CASK: Kappa-casein, LACB: Beta-lactoglobulin, ALBU: Serum albumin, ANG1: Angiogenin-1, LIPL: Lipoprotein lipase, FETUA: Alpha-2-HS-glycoprotein, BT1A1: Butyrophilin subfamily 1 member A1, TRFL: Lactotransferrin, OSTP: Osteopontin, F12AI: Factor XIIa inhibitor, CALR: Calreticulin, ACTB: Actin, cytoplasmic 1, H4: Histone H4, ACTG: Actin, cytoplasmic 2, EF1A1: Elongation factor 1-alpha 1, PERL: Lactoperoxidase, GLCM1: Glycosylation-dependent cell adhesion molecule 1, PIGR: Polymeric immunoglobulin receptor, TBA1B: Tubulin alpha-1B chain, NUCB1: Nucleobindin-1, LBP: Lipopolysaccharide-binding protein, CF136: Uncharacterized protein C6orf136 homolog, GELS: Gelsolin HS90B: Heat shock protein, FGFP1: Fibroblast growth factor-binding protein 1.

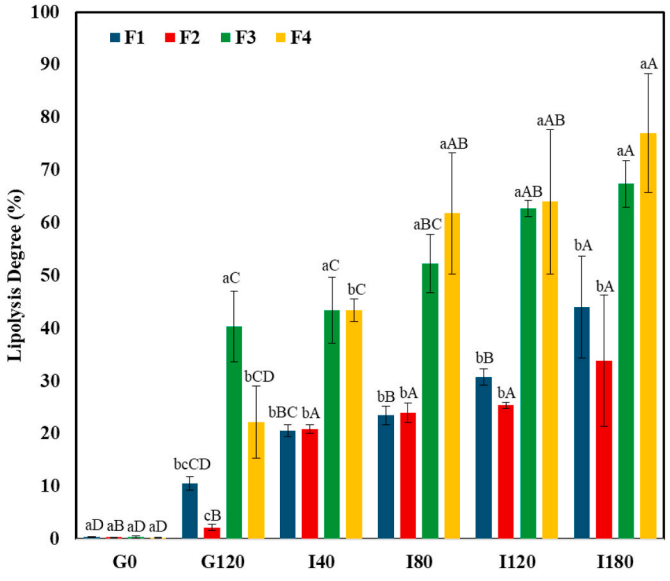

Fig. 9. Degree of lipolysis for the four IFs (G0) and in their corresponding *digesta* at different times of gastric (G120) and intestinal (I40, I80, I120 and I180) digestion. Different lowercase letters between the four IFs at the same sampling time or different uppercase letters for the same sample as time progresses indicate significant difference ($p \leq 0.05$) was revealed by the Tukey test.

the work reported in this paper.

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