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# The structure of infant formulas impacts their lipolysis, proteolysis and disintegration during *in vitro* gastric digestion

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ABSTRACT

Milk lipids supply most of the calories necessary for newborn growth in maternal milk or infant formulas. The chemical composition of infant formulas has been optimized but not the structure of the emulsion. There is still a major difference between the native emulsions of milk fat globules and processed submicronic emulsions in infant formulas. This difference may modify the kinetics of digestion of emulsions in newborns and influence lipid metabolism. To check this, semi-dynamic gastric *in vitro* digestions were conducted on three matrices: a standardized milk emulsion containing native milk fat globules referred to as minimally-processed emulsion and two processed model infant formulas (homogenized or homogenized/pasteurized). Gastric conditions mimicked those reported in newborns. The minimally-processed emulsion was lipolyzed and proteolyzed slower than processed formulas. The difference in initial structure persisted during digestion. The surface of the droplets was the key parameter to control gastric lipolysis kinetics, the pattern of released fatty acids and proteolysis by faster hydrolysis of adsorbed proteins.



## 1. Introduction

Lipid digestion is essential for adult nutrition since this macronutrient conveys calories, vitamins, hormonal precursors and membrane structural constituents. It is all the more important for infant nutrition in which milk lipids supply 50–60% of the calories necessary for newborn growth ideally under the form of maternal milk or under the form of infant formulas. Paradoxically whereas fat intake per kilogram bodyweight is three to fivefold higher in newborns compared to adults, the main actors of lipid digestion in the adults are immature in newborns (Lindquist & Hernell, 2010). The luminal concentrations of pancreatic triacylglycerol lipase and bile salts necessary for efficient intestinal hydrolysis of triacylglycerides (TAG), for the micellarization of the products of hydrolysis and efficient fat absorption are low compared to adults (Lindquist & Hernell, 2010). On the contrary, human gastric lipase (HGL) which initiates lipolysis in the gastric compartment is mature very early in the newborn (Bourlieu et al., 2014; Sarles, Moreau, & Verger, 1992).

In adult digestion, HGL is responsible for the hydrolysis of 10–30% of TAG and promotes the action of pancreatic lipase through the release of free fatty acids (FFA) which favor the anchoring of the pancreatic lipase-colipase complex at the lipid-water interface (Bakala N'Goma, Amara, Dridi, Janin, & Carrière, 2012; Lengsfeld et al., 2004). HGL could play an even crucial part in neonatal nutrition due to its early maturation and ability to attack directly native milk fat globules (Bernbäck, Blackberg, & Hernell, 1989). The physiological part played by this extremophilic enzyme relies on specific physico-chemical properties such as: a low optimal pH range (4–5) for activity, a stability to pepsin and pH denaturation above 1.5, a high tensioactivity allowing its adsorption at interfaces with low surface tension and in the presence of bile salts (Lengsfeld et al., 2004), and a stereospecificity for the *sn*-3 position of TAG (Bakala N'Goma et al., 2012; Carrière et al., 1997). Very

*Abbreviations:* CLSM, confocal laser scanning microscopy; DAG, diacylglycerol; FA, fatty acid; FFA, free fatty acid; HD, hydrolysis degree; HGL, human gastric lipase; MAG, monoacylglycerol; RGL, rabbit gastric lipase; *sn*, stereospecific numbering; TAG, triacylglycerol; TLC, thin layer chromatography; SDS, sodium dodecyl sulfate.

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similar properties are shared by the other preduodenal mammalian lipases such as rabbit gastric lipase (RGL). However none of these lipases has been commercially available up to recently, which has limited their use in physiologically pertinent *in vitro* digestion systems (Capolino et al., 2011; Chatterton, Rasmussen, Heegaard, Sorensen, & Petersen, 2004; Garcia, Antona, Robert, Lopez, & Armand, 2014). This limitation has favored the use of acid- and protease-resistant fungic lipases for replacing gastric lipase, but their equivalence to gastric lipase has never been demonstrated so far.

The composition of infant formula is based on human milk composition but the structure of these two matrices differs at different levels of scale. At the molecular level, the nature and structure of TAG on one side (Jensen, Hagerty, & McMahon, 1978), and of casein micelles and whey proteins on the other side, are not similar (Chatterton et al., 2004). At supramolecular level, the emulsions structure, *i.e.* the size of the dispersed milk fat droplets and the nature of their interface, still differs remarkably (Michalski, Briard-Bion, Michel, Tasson, & Poulain, 2005). This structure results for human milk from biological secretion and for infant formulas from a succession of technological treatments. Infant formulas are based on submicronic droplets of 0.5 μm (distribution 0.1–1 μm) whereas human milk contains bigger droplets of 4 μm (distribution 0.1–10 μm) called native milk fat globules. These native milk fat globules are stabilized by a trilayered membrane inherited from their secretory pathway in the mammary gland. The membrane has a typical composition of mammalian cell membrane (phospholipids, sphingolipids, cholesterol, proteins and enzymes) (Lopez, Madec, & Jimenez-Flores, 2011). On the contrary, fat droplets in formulas are stabilized by a neoformed membrane based on caseins, whey protein aggregates and possibly remnants of milk fat globule membrane. Recent studies suggest that these differences in supramolecular structure of emulsions between human milk and infant formulas are key elements accounting for the major long-term health benefits of breastfeeding, such as protection from metabolic syndrome and obesity (Oosting et al., 2014). In parallel to these systemic effects, the luminal phase of milk lipids digestion is very likely to be affected by the difference of structure between native and processed dairy emulsions. It is well established that the rate of lipolysis is controlled by the main physicochemical characteristics of the oil/water interface: the surface area of the lipid droplets (Benzonana & Desnuelle, 1965) and their interfacial composition (Verger & de Haas, 1976). Macierzanka, Sancho, Mills, Rigby, and Mackie (2009) also demonstrated that the adsorption of caseins and β-lactoglobulin at a hydrophobic interface favors their susceptibility to pepsin proteolysis.

Lipolytic and proteolytic processes are therefore highly dependent on the structure of food emulsion, which can be considerably altered by the environment and physicochemical conditions encountered in the gastro-intestinal tract (Gallier, Ye, & Singh, 2014; Singh, Ye, & Horne, 2009). Similarly, the initial structure of infant formula resulting from technological treatments may seriously impact gastric lipolysis and proteolysis. A very recent study (Garcia et al., 2014) has compared the lipolysis extent of milks containing native or homogenized milk fat using a static *in vitro* digestion model mimicking adult gastric digestion and concluded that homogenization did not affect the gastric lipolysis extent (9% hydrolysis of TAG). However, the study did not compare directly batches of milk having the same composition nor established the kinetics of gastric lipolysis.

The purpose of the present study was to determine to what extent homogenization and heat treatment had an impact on the gastric hydrolysis and disintegration of infant formulas. The results of this study could be useful to food scientists and infant food manufacturers as it highlights the influence of the structure of the emulsion on the luminal part of neonatal gastric digestion.

In this study, three model infant formulas, based on bovine milk and representative of native, homogenized or homogenized and heat-treated emulsions were formulated. The model formulas were then subjected to *in vitro* digestion using a semi-dynamic model mimicking the infant gastric phase.

## 2. Materials and methods

### 2.1. Materials

Unless otherwise stated, chemicals were from commercial origin (Sigma–Aldrich, Saint-Quentin Fallavier, France).

### 2.2. Preparation of model infant formula emulsions

Raw cow's milk (20 L) was purchased from a local dairy plant (Entremont, Montauban de Bretagne) and transformed into three model infant formulas with similar chemical composition but three distinct structures. At reception milk was submitted to a minimal heat treatment to inactivate endogenous lipase (54 °C, 20 min) without denaturing milk proteins (Deeth, 2006). Milk was then skimmed (elecrem, type 125, Châtillon, France; rate: 315 L/h 45 °C) and standardized at 3.3% fat content and at 2.1% protein content by redispersion of the cream in the skim milk and dilution of the skim milk with milk ultrafiltrate (5 kDa). The casein/whey protein ratio was readjusted from its initial value of 80:20 w/w to 40:60 w/w by addition of milk whey protein concentrate (Prolacta®95).

The matrix obtained after this standardization was called M1 and can be considered as 'minimally processed' as it was composed of native milk fat globules dispersed in a standardized colloidal phase whose protein composition mirrored that of infant formulas. The preservation of the milk fat globules after skimming was checked by apparent zeta-potential assessment in the experiments (Zetasizer Nano series Nano-ZS, Malvern Instruments, Malvern, UK): the apparent zeta-potential of the raw milk dispersed in a buffer mimicking milk ionic strength (pH 7, 5 mM $CaCl_2$, 50 mM NaCl, 20 mM imidazole; Dalgleish, 1984) was −10.5 (0.5); the apparent zeta-potential of the cream redispersed in the same buffer was −10.1 (0.7) indicating that the skimming had been performed gently and preserved the milk fat globule membrane. Other experimental evidence was collected indicating that the milk fat globules in M1 were preserved: – granulometry analysis did not present a range of particles of larger size which would have been formed by aggregation during skimming, – confocal microscopy observations revealed individual fat globules surrounded by intact membrane without aggregation or coalescence, – lipolysis experiments were also conducted on heat-treated (54 °C, 20 min) milk without skimming nor standardization and gave very similar rate of free fatty acids release indicating that the minimal treatments applied to process M1 (skimming and standardization) had minimal impact on native milk fat globules. The second matrix M2 was obtained by homogenization of M1 using a two-stage high-pressure homogenizer at 220-20 bars (Rannie slow model LAB 16/15, APV France, Evreux, France). Eventually a pasteurization treatment (72 °C, 15 s) was applied to M2 to obtain the matrix M3.

Two batches of raw milk were purchased and processed to obtain the three matrices in duplicate. Their main macronutrients composition with regards to fat [%], protein [%] and lactose [%] was respectively: 4.33 ± 0.00, 3.50 ± 0.01, 4.89 ± 0.02 for the first batch of initial milk; 4.25 ± 0.00, 3.46 ± 0.01, 4.91 ± 0.02 for the second batch of initial milk; 3.27 ± 0.01, 2.08 ± 0.00, 4.72 ± 0.01 for the first batch of model emulsion and 3.26 ± 0.00, 2.20 ± 0.01, 4.65 ± 0.02 for the second batch of model emulsion.

### 2.3. Semi-dynamic in vitro digestion of model infant formulas

#### 2.3.1. In vitro device

Simulated digestions were conducted in hermetic thermoregulated glass vessels (37 °C ± 0.5 °C) of a pH-stat device (Titrando 842, Metrohm SA, Courtaboeuf, France). The initial meal volume was 25 mL.

#### 2.3.2. Selection of parameters to mimic infant gastric conditions

To closely mimic the physiological processes that occur during infant gastric digestion, the decrease of pH, the level of dilution of the meal by gastric secretions (simulated gastric fluid and titrant) and the added quantity of digestive enzymes were adjusted according to the *in vivo* data (Bourlieu et al., 2014; Roman et al., 2007). The pH-stat device was programmed to established three subsequent stages at pH 6 (0–60 min), pH 5 (60–120 min), pH 4 (120–180 min) (see Supplementary data Fig. 1 for curves). At the beginning of each stage, the pH was adjusted by addition of a simulated gastric fluid (HCl 150 mM, NaCl 110 mM, $CaCl_2$ 5 mM). The secretions resulted in a dilution of the meal by 30% v/v during the entire digestion. The amount of NaOH required to maintain the equilibrium was used to calculate the concentration of titratable free fatty acids (FFA) released during the hydrolysis and corrected considering FA average theoretical pKa of 4.75. The corresponding hydrolysis degree was expressed as the percentage of FFA released from the total acyl moieties initially esterified in TAG in the emulsion and calculated as follows:

$$HD = \frac{FFA \cdot MMeq}{FC \cdot V \cdot [TAG] \cdot 3} \quad (1)$$

With HD: hydrolysis degree in % of acyl moieties initially esterified as TAG, FFA: free fatty acids in mol, MMeq: average TAG molar mass of 794 g $mol^{-1}$ deduced from GC analysis of fatty acids, FC: average fat concentration in g $mL^{-1}$, V: volume of emulsion in the pH stat vessel, [TAG]: theoretical TAG content of 98.5% in total milk lipids.

It is important to note that this direct titration corresponded to the exclusive release of FA in the presence of RGL only whereas in the presence of RGL and pepsin some free amino acids released by pepsin could also contribute to the titration. Thus, in the presence of RGL and pepsin, results were consolidated by systematic comparison with the HD estimated from IATROSCAN lipid analysis.

Animal enzymes were used to mimic human enzymes present in infants: rabbit gastric lipase (RGL) presenting 84% homology with human gastric lipase (HGL) was selected in agreement with the recent work of Capolino et al. (2011) which validated RGL as a good substitute of HGL. RGL was purified as previously described (Moreau, Gargouri, Lecat, Junien, & Verger, 1988) and mixed with adjuvants before lyophilization. The resulting powder had a specific activity of 169 U $mg^{-1}$ (1 U = 1 μmol of FFA released per minute) under standard assay conditions of human gastric lipase (pH 6, 37 °C) using tributyrin as substrate, (Gargouri et al., 1986) which corresponds to 25% w/w of lipase. Porcine pepsin was used as a substitute of Human gastric pepsins (Sigma–Aldrich, Saint-Quentin Fallavier, France). Pepsin activity was measured by the hemoglobin method as detailed in Ménard et al. (2014) and found equal to 3 100 U; one unit will produce a deltaA 280 nm of 1 μmol per minute at pH = 2 and 37 °C, measured as trichloracetic acid soluble products. In agreement with the averaged lipolytic and proteolytic activities detected in newborn gastric contents (Armand et al., 1996; Roman et al., 2007), respectively 26 U.$mL^{-1}$ of initial gastric content of lipase and 63 U.$mL^{-1}$.$kg^{-1}$ of infant bodyweight (considering an average weight 4.15 kg) of pepsin powder were added at the beginning of the digestion.

#### 2.3.3. Experimental planning of in vitro digestions

Three types of digestion were conducted: (1) a control digestion without digestive enzymes to dissociate the effect of the enzyme activity from the effect of the pH decrease on the structure of the matrix; (2) a digestion with RGL only, and (3) a digestion with RGL and pepsin to allow the comparison of additional proteolytic effects. The 3 types of digestion were conducted at least in duplicate for each kind of model emulsions of batch 1 and batch 2.

#### 2.3.4. Aliquots during in vitro digestions

At initial time, 30, 60, 120 and 180 min, aliquots (1.5 mL) were collected. Some were directly used for particle size measurements and microscopic observations. Other aliquots were mixed with stop solutions whose compositions are detailed below, frozen (−20 °C) and used for subsequent lipid class analysis or protein analysis.

### 2.4. Chemical characterization of infant formula emulsions during the digestion

Three complementary analyses were conducted on the aliquots withdrawn from the digestion: analysis of lipid classes (residual TAG, DAG, MAG and total FFA) by thin layer chromatography (TLC) and IATROSCAN®, analysis of FFA released during the digestion by gas chromatography and analysis of proteins by electrophoresis.

Briefly, 200 μL of sample were collected for TLC analysis, mixed with 1.2 mL of chloroform/methanol (2:1 v.$v^{-1}$) and acidified with 80 μL of HCL 0.1 N to stop the reaction. The extract was then rinsed with NaCl 0.73% (48 μl) and 300 μL of supplementary chloroform/methanol (2:1 v.$v^{-1}$). The chloroformic phase was spotted on silica gel plates (10 × 20 cm, 0.25 mm, Si G60, Merck) using Automated TLC Sampler III (CAMAG, Muttenz, Switzerland) as detailed in Bourlieu, Rousseau, Briard-Bion, Madec, and Bouhallab (2012). This qualitative characterization was reinforced by a quantitative characterization of lipid classes by IATROSCAN analysis corresponding to TLC coupled to a flame ionization detector.

FFA (C4:0 to C18:3) were analyzed by gas chromatography (GC) using three internal standards (80 μL of C5, C11 and C17 at 0.5 mg $mL^{-1}$ added to aliquot prior solvent extraction) as previously described by Bourlieu et al. (2012). The extractions were conducted in duplicate for each sample.

SDS–PAGE was performed on 200 μL aliquots withdrawn from digestion inhibited with PMSF using 4–12% polyacrylamide NuPAGE Novex *bis*-Tris 15-well precast gels (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and as described previously by Bouzerzour et al. (2012) except that the samples were not centrifuged prior to analysis. Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight (Mw) marker. Gels were then fixed in 30% (v.$v^{-1}$) ethanol, 10% (v.$v^{-1}$) acetic acid and 60% (v.$v^{-1}$) deionized water and were rinsed in deionized water before staining with Coomassie Blue. Image analysis of SDS–PAGE gels was carried out using Image scanner III (GE Healthcare Europe GbmH, Velizy-Villacoublay, France) and Magicscan software.

### 2.5. Structural characterization of infant formula emulsions during the digestion

#### 2.5.1. Particles size measurements

The size distributions of fat globules in the model emulsions were measured by laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK), with two laser sources as previously described in Bourlieu et al. (2012). From the size distribution, several parameters were calculated: the mode diameter, which corresponds to the population of the fat globules the most

frequent in the volume distribution; the surface-weighted average diameter of fat globules $d_{32}$ (μm), defined as $\Sigma n_i d_i^3/\Sigma n i d i^2$; and the specific surface area calculated according to the equation $S = 6.\phi/d_{32}$ (where $\phi$ is the volume fraction of lipid in the emulsion).

*2.5.2. Confocal laser scanning microscopy (CLSM)*

The matrix microstructure was observed using a Nikon C1Si Laser Scanning Confocal Imaging System on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) operated with an argon laser (excitation at 488 nm) and two He–Ne lasers (excitation at 543 and 633 nm) which allowed multi-imaging of a sample by selecting the correct excitation wavelength and filters to collect the emission light from a particular stain. A 60 × oil-immersion objective was used for all images. Three fluorescent dyes were used to localize respectively (i) proteins with Fast green FCF (6:100 v.v$^{-1}$, λex 633 nm – λem 655–755 nm, Invitrogen), (ii) apolar lipids with Lipidtox® (0.2:100 v/v, λex 488 nm – λem 590 nm, Invitrogen) and (iii) polar lipids with Rd-DOPE® (1:100 v/v, 16:0 Liss Rhod PE *1,2*-dipalmitoyl-*sn*-glycero-3-phosphoethanolamin-N-(lissamine rhodamine B sulfonyl, λex 543 nm – λem 590 nm, Aventi polar lipids). The three dyes were added in 200 μL digestion aliquots at least 10 min before observation. The sample (10 μL) was then applied on a glass slide and fixed by delicate mixing with 5 g L$^{-1}$ low melting point agarose (Sigma–Aldrich, St. Quentin Fallavier, France) just before the observations. The software used for CSLM images was EZ-C1 version 3.40 (Nikon).

*2.6. Statistics*

All results are presented as mean ± SD. Statistical significance between the matrices was tested by one way ANOVA using R software (R.2.13.0, http://cran.r-project.org). Differences between groups were declared significant at $P < 0.05$.

## 3. Results

*3.1. The initial structure of fat droplets affected the kinetics of matrix hydrolysis*

*3.1.1. Lipolysis of matrices in the presence of RGL alone*

The levels of FFA released during the hydrolysis of the matrices by RGL alone and titrated by pH-stat are displayed in Fig. 1A. The kinetics of hydrolysis of TAG were significantly influenced by the type of matrix ($P < 0.01$) from 0 to 120 min. Over this period of time, the rates of lipolysis are classified as follows: rate of minimally-processed matrix M1 << rate of the homogenized matrix M2 < rate of the homogenized-pasteurized matrix M3. For the two most processed matrices, most of the hydrolysis (62–64%) occurs during the first 30 min. The corresponding initial rates of hydrolysis were estimated over the first 30 min of the lipolysis reaction ($V_{T0-T30min}$; Table 1). Based on these initial rates $V_{T0-T30min}$ the same classification for the lipolysis of the matrices was established: M1 << M2 < M3 with rates respectively 19 times higher for M2 and 25 times higher for M3 as compared to M1. This lower rate of lipolysis of matrix M1 has to be put in relation with the difference in initial particle size distribution compared to the two most processed matrices (Fig. 1C). The native milk fat globules present in M1 have a unimodal distribution with a maximum at 4 μm whereas the most processed matrices present a bimodal distribution centered on 0.2–0.6 μm. As a result, the lipid phase of M1 develops a much reduced specific surface of 1.8 m$^2$ g$^{-1}$ of lipid compared to the high specific surface developed by the neoformed lipid droplets dispersed in the processed matrices M2 and M3 (31.7–32.0 m$^2$ g$^{-1}$ of lipids) (Table 1). When normalizing the initial rate of lipid hydrolysis of the matrices by their respective initial specific surface, ratio $V_{T0-T30min}$/SS, all the matrices presented were close and showed non-significantly different initial rates of lipolysis of ~0.1–0.2 μmol min$^{-1}$ m$^{-2}$ of lipid surface.

With regard to the extent of the lipolysis during the 180 min of the simulated gastric phase, the hydrolysis degree (HD) determined by pH-stat is limited for all the matrices, ranging from 4.6% (M1) to 11% (M3) hydrolysis of total ester bonds of TAG initially present in 25 mL emulsion, *i.e.* 3.03 mM of initially esterified FA. Again the minimally-processed matrix M1 displayed the lowest HD (4.6 ± 2.4%), though not significantly different from the homogenized matrix M2 (HD = 7.7 ± 1.9%) whereas M3 is the most extensively hydrolyzed (11.0 ± 0.9%) over the 180 min of digestion.

The profile in released fatty acids characterized by GC in comparison with the profile of total fatty acid initially esterified in the matrices is displayed in Fig. 2A. This profile was marked by high levels of short chain (C4–C8) and medium chain fatty acids (C10) and lower ones in palmitic (C16:0), myristic (C14:0) and oleic acids (C18:1 *cis* 9) at the beginning of the digestion of M2 and M3, while the release of short (C6–C8) to medium chain (C10) fatty acids was delayed in M1 in relation with its slower initial lipolysis. The initial profile of released fatty acids from M1 was dominated by two long chain fatty

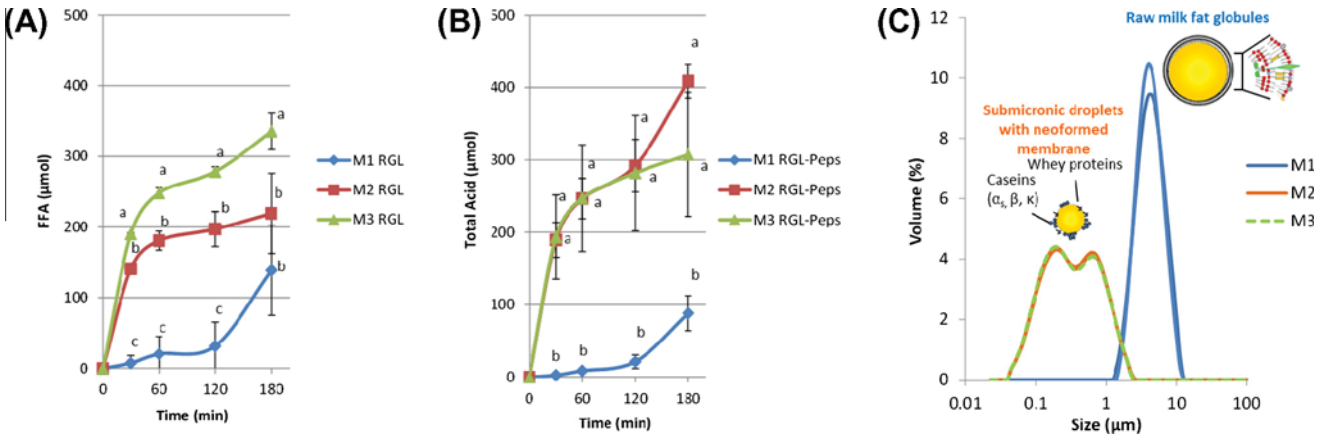



**Fig. 1.** Kinetics of lipolysis of the three matrices M1, M2 and M3. (A) FFA released during *in vitro* gastric digestion of the 3 matrices in the presence of RGL only. The titrations were made by pH-stat. Duplicates at least on each of the two batches of milk; (B) Total acid released during *in vitro* gastric digestion of the 3 matrices in the presence of RGL and pepsin. The titrations were made by pH-stat. Duplicates at least on each of the two batches of milk; (C) comparison of the initial lipid particles size distribution of the three matrices measured by light scattering with addition of EDTA. Different letters between matrices at a given time indicate significant difference using one way ANOVA test ($P = 0.05$).

**Table 1**
Initial rates of lipolysis, with or without normalization by the emulsion initial specific surface, and hydrolysis degree (HD) of the three matrices after 180 min during *in vitro* gastric digestions in the presence of RGL only or of RGL and pepsin (RGL-peps).

| | M1-RGL | M2-RGL | M3-RGL | M1-RGL-peps | M2-RGL-peps | M3-RGL-peps |
|---|---|---|---|---|---|---|
| $V_{T0-T30min}$ [μmol min$^{-1}$] | 0.25 ± 0.36$^{a}$ | 4.70 ± 0.20$^{b}$ | 6.34 ± 0.05$^{c}$ | 0.09 ± 0.07$^{a}$ | 6.31 ± 0.80$^{b,c}$ | 5.40 ± 1.98$^{c}$ |
| Specific surface [m$^2$ g$^{-1}$ of lipid] | 1.81 ± 0.02$^{a}$ | 31.65 ± 0.35$^{b}$ | 32.03 ± 0.31$^{b}$ | 1.81 ± 0.02$^{a}$ | 31.90 ± 0.20$^{b}$ | 32.12 ± 0.00$^{b}$ |
| $V_{T0-T30min}$/SS [μmol min$^{-1}$ m$^{-2}$] | 0.14 ± 0.20$^{a}$ | 0.15 ± 0.01$^{a}$ | 0.20 ± 0.00$^{a}$ | 0.11 ± 0.04$^{a}$ | 0.20 ± 0.03$^{a}$ | 0.18 ± 0.07$^{a}$ |
| HD [% ester] – pH-stat | 4.6 ± 2.1$^{a,c}$ | 7.2 ± 1.9$^{a}$ | 11.0 ± 0.9$^{b}$ | 2.9 ± 0.8$^{c}$ | 13.5 ± 0.8$^{d}$ | 10.1 ± 2.8$^{b,d}$ |

[a]Different letters on a same line stand for significant difference using one way ANOVA test ($P = 0.05$).

acids:stearic (C18:0) and oleic acids (C18:1 *cis* 9). During the course of hydrolysis, the quantity of short to medium chain fatty acids (C6–C10) and of myristoleic acid (C14:1 *cis* 9) increases at the expense of stearic acid. The proportion of palmitic acid in the free fatty acids remained much lower 7.5–8.5% than in the total fatty acids initially esterified, *i.e.* 37.5%.

The global evolution of the classes of lipids (TAG, DAG, MAG, FFA) is reported in Fig. 2B. As expected, the classes of lipids were dominated by residual TAG and two types of products of lipolysis FFA and *sn*-1,2(2,3) DAG for the three matrices. From 30 min onwards, traces of MAG appeared in the two most processed matrices indicating that a small fraction of *sn*-1,2(2,3) DAG are further hydrolyzed to MAG in the gastric phase.

### 3.1.2. Coupled in vitro lipolysis and proteolysis of the matrices

To determine the impact of pepsin during neonatal gastric digestion, pepsin (261 U.mL$^{-1}$ gastric content) was added in the *in vitro* digestion model. Pepsin had limited impact on the initial rate of lipolysis catalyzed by RGL as displayed in Fig. 1B. The initial rate of lipolysis $V_{T0-T30min}$ did not significantly differ in the presence of pepsin for any of the three matrices (Table 1). The addition of pepsin however, cancelled the difference of lipolysis kinetics between the two most processed matrices M2 and M3, by increasing the release of fatty acids from the homogenized matrix M2. This increase in lipolysis for M2 resulted in a significantly higher HD in the presence of pepsin (13.5 ± 0.8%) than without pepsin (7.2 ± 0.8%) after 180 min of incubation, and overall, M2 became a better substrate than M3. Since the pH-stat titration results could be biased by the detection of some free amino acids released by pepsin, the titration results were consolidated by systematic comparison with the HD estimated from IATROSCAN lipid analysis. HD estimated from IATROSCAN of 2.4% for M1, 13.2% for M2 and 12.6% for M3 were all in agreement with the results obtained by pH-stat titration. The distribution of lipid classes present in the system during digestion was

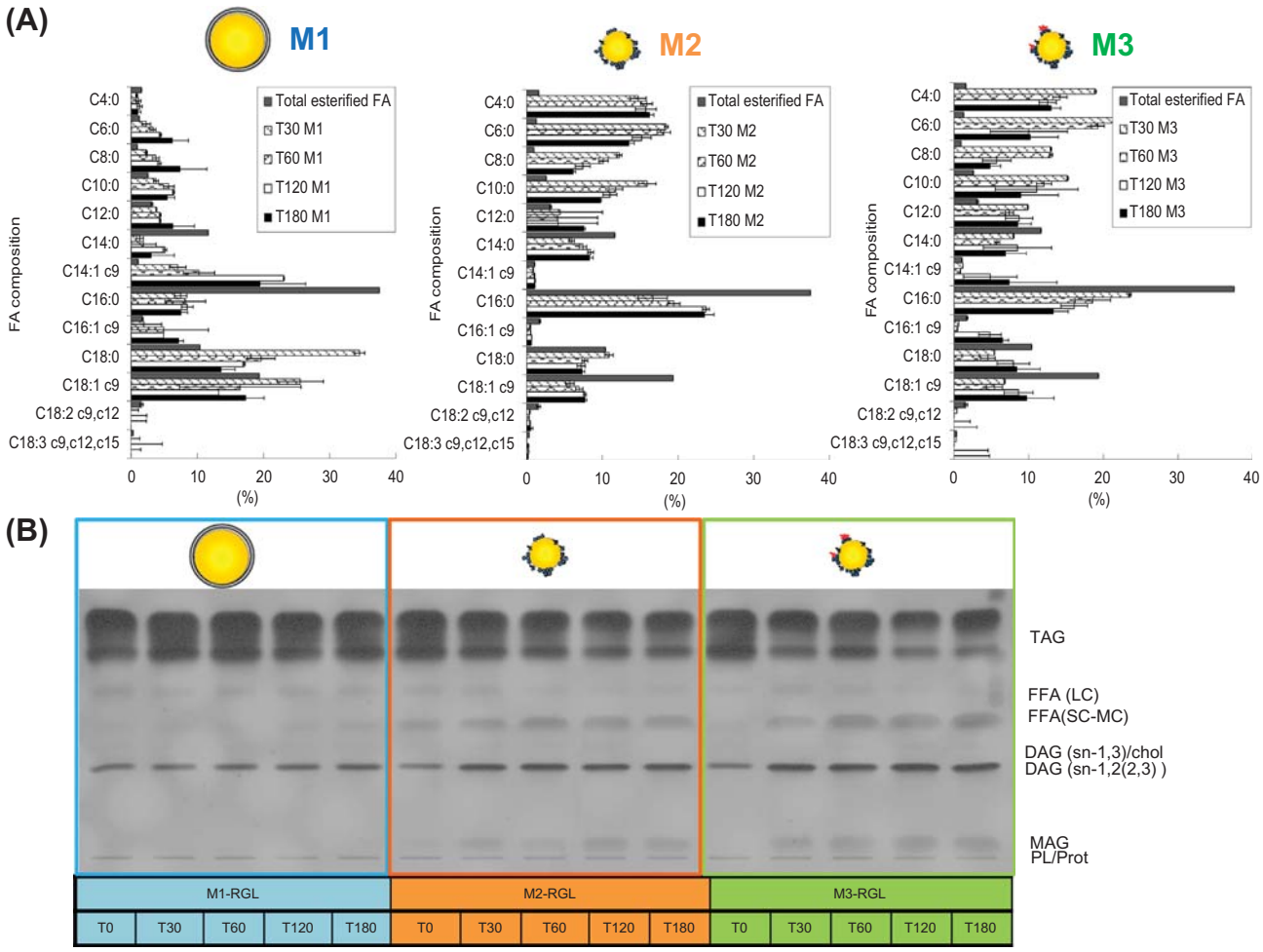



**Fig. 2.** Products of lipolysis of the three matrices M1, M2 and M3. (A) GC Profile of FFA released from the matrices in comparison with total esterified fatty acids in TAG. (B) Example of thin layer chromatography showing the evolution of lipid classes during the *in vitro* gastric digestions of the 3 matrices in the presence of RGL only. *Abbreviations.* TAG: triacylglycerol, FFA: free fatty acids, LC: long chain, SC: short chain, DAG: diacylglycerol, chol: cholesterol, MAG: monoacylglycerol, PL: polar lipid, prot: protein.

not modified by the addition of pepsin (Supplementary data Fig. 2) and consisted mainly in TAG, medium to short chain FFA, *sn*-1,2(2,3) DAG and a small quantity of MAG.

With regard to the proteolysis catalyzed by pepsin, SDS–PAGE analysis is reported in Fig. 3. It indicated that caseins ($\alpha_{s1}$–$\alpha_{s2}$, β and κ) were hydrolyzed faster than whey proteins (β-lactoglobulin and α-lactalbumin). A matrix effect was observed in the proteolysis of caseins with an initial faster disappearance of $\alpha_{s1}$–$\alpha_{s2}$, β and κ caseins in M2 and M3 compared with M1: 41–49% of the initial caseins were hydrolyzed in 60 min compared to 22% only in M1. The hydrolysis of the caseins followed a first order reaction with $k = 0.02\ min^{-1}$ for M2 and M3 ($r^2 = 0.97$ and 0.96 resp.) and $k = 0.01\ min^{-1}$ for M1 ($r^2 = 0.94$). Whatever the matrices, most of the caseins, accounting for 40% of the total proteins in the matrices, had disappeared within the 180 min of gastric digestion (Fig. 3C). β-Lactoglobulin was totally resistant over the gastric phase (Fig. 3D) whereas α-lactalbumin was hydrolyzed to a level of around 20% only during the last period (120–180 min). This period corresponds to the lowest pH value (pH 4) and therefore the most favorable for pepsin activity.

### 3.2. The initial structure affected the disintegration of the matrices

#### 3.2.1. Impact of the pH decrease on emulsion colloidal structure

The impact of the stepwise pH decrease (pH 6 from 0 to 60 min, pH 5 from 60 to 120 min and pH 4 from 120 to 180 min) on the particle size distribution is summarized in Fig. 4. The decrease of pH induced the aggregation of lipid droplets and proteins but at different rates depending on the matrix: M1 was the most resistant to acid aggregation with a stability of particle size up to 120 min of digestion, M2 was already destabilized at 120 min, and M3 was destabilized as soon as 30 min. The mode(s) or most frequent size in the volume distribution at 180 min was affected by the initial matrix structure with a larger mode for M1 (161 μm), an intermediate for M2 (92 μm) and a smaller one for M3 (50 μm). The addition of sodium dodecyl sulfate (SDS), an anionic surfactant, after 180 min restored a particle size distribution very close to the initial distribution. The structure of the aggregates investigated by CLSM is displayed in Fig. 5 (A, B, C Timelines M1-C, M2-C and M3-C). The native milk fat globules of M1 remained dispersed in the colloidal phase of the matrix up to 120 min. This colloidal phase is based on dispersed caseins and soluble whey proteins both colored in blue by FastGreen®. The typical structure of the milk fat globules characterized by an hydrophobic core of TAG and other esters (labeled by Lipitdox® in green) and enveloped by their natural membrane with liquid ordered domains which do not fix the red-labeled Rhodamine-PE® was very clearly visualized on the 5× magnification (T60 min). Interestingly this structure was maintained even when the matrix aggregated at 180 min either as dispersed elements or inside large mixed aggregates of proteins trapping clusters of milk fat globules. In the two most processed matrices (B M2-C and C M3-C), the initial droplets with neoformed droplets of smaller sizes (0.2–0.6 μm) formed a background in which the apolar core of the droplets is labeled in green. These droplets are trapped in mixed aggregates of proteins and lipids whose sizes progressively increased from 120 up to 180 min in M2-C and from 30 min up to 180 min in M3-C.

#### 3.2.2. Impact of enzymes

The addition of RGL sped up the matrices aggregation in M1 and M3 (Fig. 4B). On the contrary, the addition of lipase tended to limit the size of the aggregates in M2. Whatever the matrices, pepsin

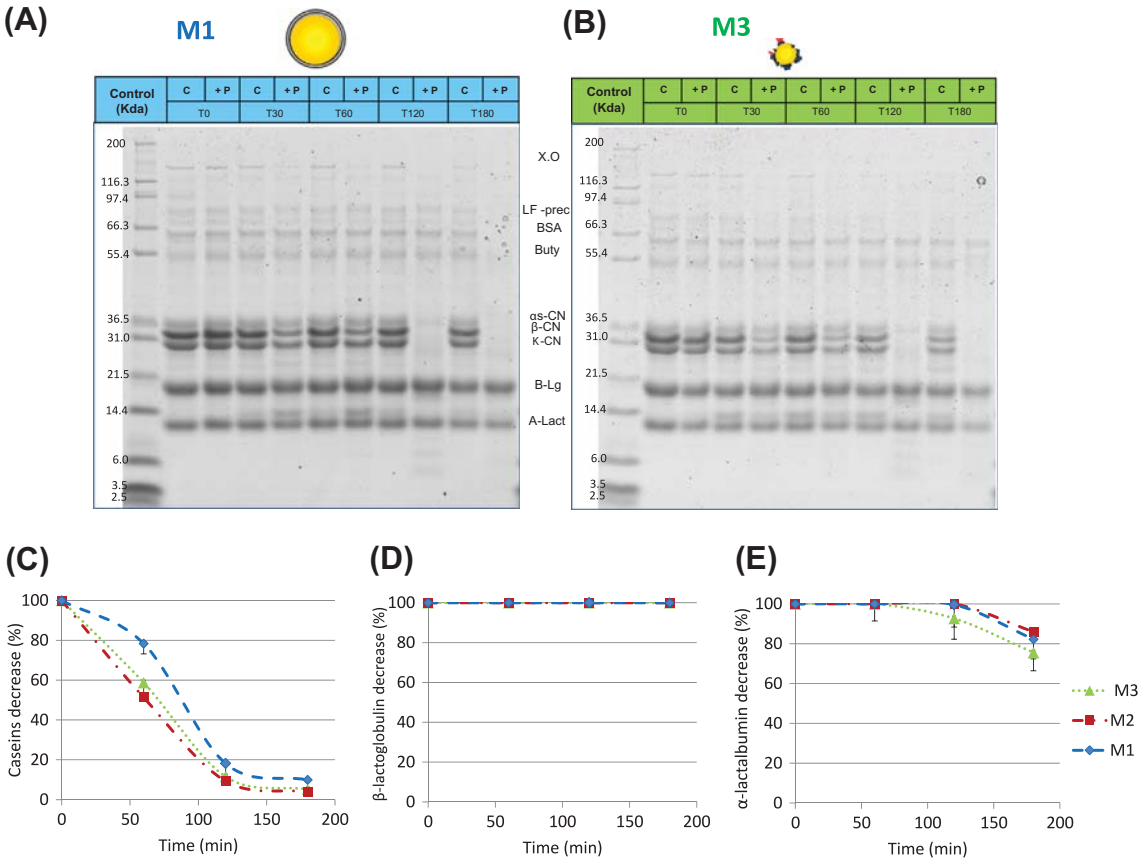
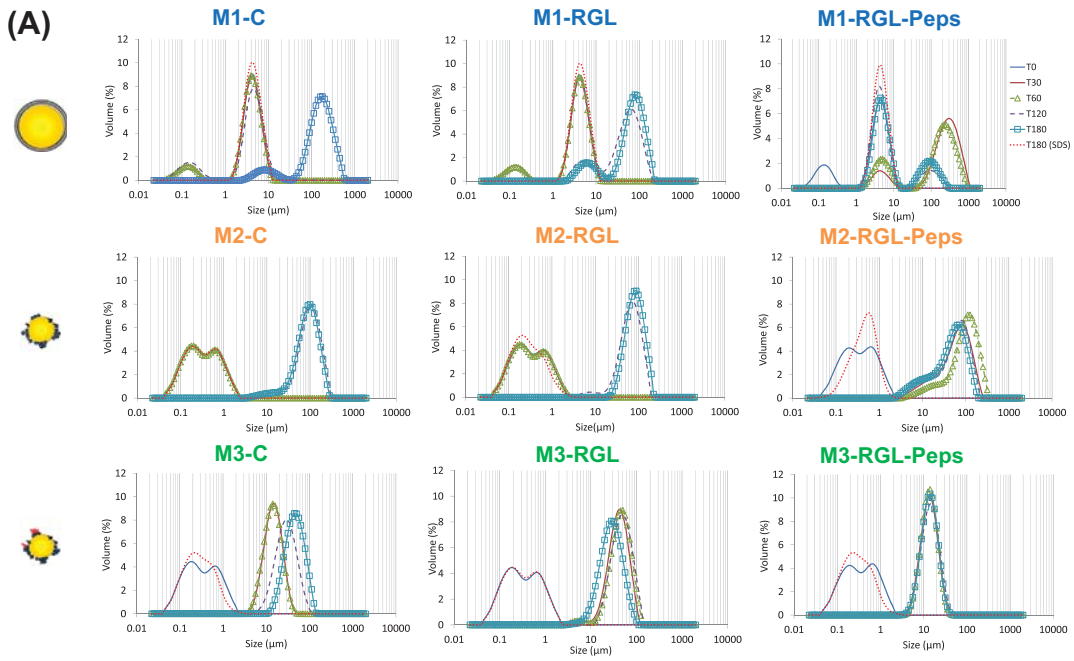



**Fig. 3.** SDS–PAGE analysis of the evolution of proteolysis during *in vitro* gastric digestion of M1 (A) in comparison with M3 (B) in the presence of RGL and pepsin. As M2 and M3 SDS–PAGE gels showed very similar trends, M3 gel was presented only. Corresponding kinetics of proteolysis obtained by densitometry: (C) for caseins, (D) for β-lactoglobulin, (E) for α-lactalbumin.




(B)

| Evolution of mode [µm] with time [min] | 0 min | 30 min | 60 min | 120 min | 180 min |
|---|---|---|---|---|---|
| M1-C | 4.0-0.1 | 3.9-0.1 | 3.9-0.1 | 4.3-0.1 | 161-7.5 |
| M1-RGL | 4.0-0.1 | 3.8-0.1 | 3.8-0.1 | 87.8-3.6 | 123.8-35.8 |
| M1-RGL-Peps | 4.0-0.1 | 190.0-4.2 | 141.7-4.1 | 4.0-70.9 | 4.3-67.1 |
| M2-C | 0.2-0.6 | 0.2-0.6 | 0.2-0.6 | 100.1-13.8 | 92.4 |
| M2 RGL | 0.2-0.6 | 0.2-0.6 | 0.2-0.6 | 23.0-2.8 | 58.0 |
| M2 RGL-Peps | 0.2-0.6 | 91.9 | 110.3 | 81.4 | 61.6 |
| M3-C | 0.2-0.6 | 7.1 | 6.6 | 44.4 | 50.2 |
| M3-RGL | 0.2-0.6 | 13.5-2.0 | 20.9-1.8 | 58.6-6.0 | 55.4-6.1 |
| M3-RGL-Peps | 0.2-0.6 | 31.8-2.9 | 37.5 | 32.9 | 30.1 |

Blue background indicates aggregation

**Fig. 4.** (A) Evolution of particles size distribution of the three matrices during *in vitro* gastric digestions in control without enzyme (C), with RGL only (RGL) and with RGL and pepsin (RGL-peps). Average of duplicates on one batch of milk. The particles size distribution measured after addition of sodium dodecyl sulfate (SDS) at 180 min is indicated by a red dotted line. (B) Evolution of the mode(s) of the matrices (M1, M2 and M3) during *in vitro* gastric digestions. The modes were measured by laser light scattering in water. Average values are presented. Blue background indicates aggregation. Two figures separated by a dash stand for bimodal distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was a highly destabilizing agent inducing the aggregation of particles at pH 6 and within the first 30 min of the reaction. In M1 and M2, pepsin gave rise within 30 min to large aggregates, with modes of resp. 190-4.2 (bimodal distribution) and 92 µm (unimodal distribution). In M3, the addition of pepsin resulted in larger aggregates at 30 min than in the presence of RGL only (bimodal distribution, modes of 31.8–2.9 µm *vs* 13.5–2.9 µm). This trend was maintained at 60 min. Afterwards, on the contrary, the size of the aggregates were smaller and more homogeneous than in the absence of the protease (~30 µm, unimodal distribution, *vs* ~55–6 µm bimodal distribution).

### 3.2.3. Persistence of initial structural elements

The kinetics of gastric aggregation and internal structure of the aggregates were easily visualized on CLSM images (Fig. 5). Magnifications (Zooms 2–6, Fig. 5D) and decompositions of the image (with or without protein labeling) revealed the persistence of preserved milk fat globule in M1 or neo-formed lipid droplets in M2 and M3. These lipid particles were the core elements around which protein aggregates built up. Amphiphilic compounds labeled with Rd-Phosphatidylethanolamine tended to accumulate on the surface of the protein aggregates. The quasi-restoration of initial particle size distribution in the presence of dissociating agent (SDS) was another indication of the persistence of initial lipid particles as building blocks of the aggregates (Fig. 4A, dotted red lines).

## 4. Discussion

This study aimed at determining to which extent two common technological processes used to prepare and stabilize infant formulas, homogenization and heat-treatment, modify their gastric hydrolysis and desintegration. We evidenced clearly that:

(i) The rate of lipolysis were significantly lower for the minimally-processed matrix M1 compared to processed matrices: 'homogenized' M2 or 'homogenized/pasteurized' M3. Similarly a lower degree of hydrolysis (HD) was obtained for M1 compared to the most transformed matrices after 180 min gastric digestion (M2 and M3 with and without pepsin).
(ii) Rates of casein proteolysis were lower in M1 compared to the two most processed matrices.
(iii) Kinetics of gastric aggregation and internal structure of the aggregates differed between the three matrices.

These differences in gastric behaviors are discussed below, in the light of the biophysical and biochemical properties of the matrices and of gastric lipase. Potential implications on infant digestive physiology and lipid metabolism is also commented.

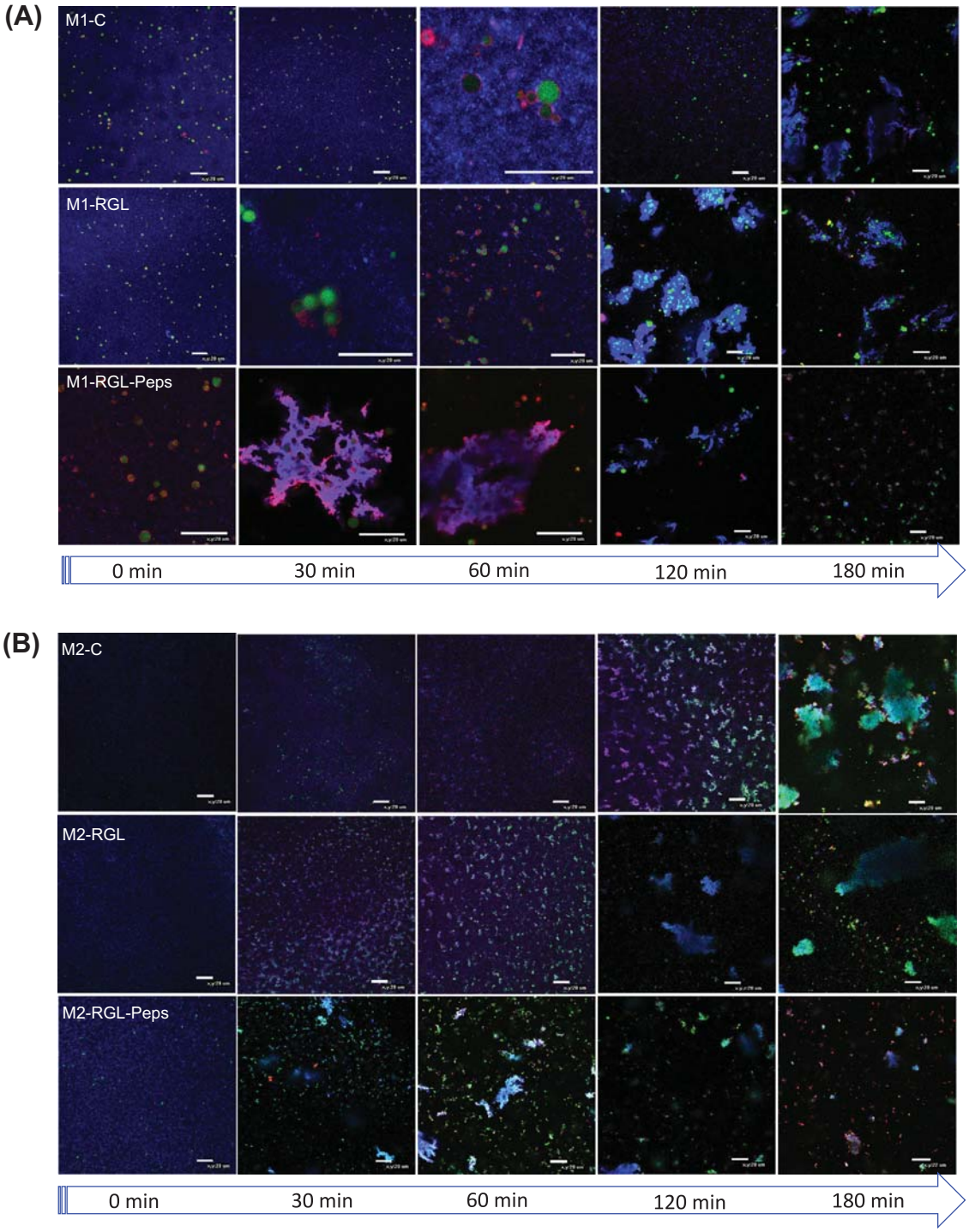



**Fig. 5.** Confocal micrographs of the natural emulsion during *in vitro* gastric digestions control without enzyme (C), with RGL only (RGL) or with RGL and pepsin (RGL-peps). Legend: (A) M1, (B) M2, (C) M3, (D) details of the gastric aggregates observed at the end of the simulated digestion (T 180 min, row 1: protein, apolar lipids and polar lipids staining, row 2: apolar lipids and polar lipids staining only). Each frame is the average of 3 observations, ×60 zoom 1–6. Proteins are colored in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE®). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### *4.1. Biochemical and biophysical basis for the difference of susceptibility to lipolysis of the three matrices*

The initial rates of lipolysis once normalized by the initial specific surface developed by the lipid phase of the emulsion did not differ significantly between the three matrices. This similarity indicates that the matrix-dependent kinetics of lipolysis observed are mainly governed by the size of the lipid emulsion droplets and the resulting specific surface accessible to the lipase. After the first 30 min of digestion, the surface of the lipid-water interface is reduced due to acid and enzyme-driven aggregation in some of the matrices in the presence of RGL only and for all the matrices in the presence of RGL and pepsin. Nevertheless, this structural reorganization did not modify the initial trend observed during the first 30 min of digestion. The slowing down in the lipolysis of M2 and M3 is more likely linked to lipolysis products inhibition

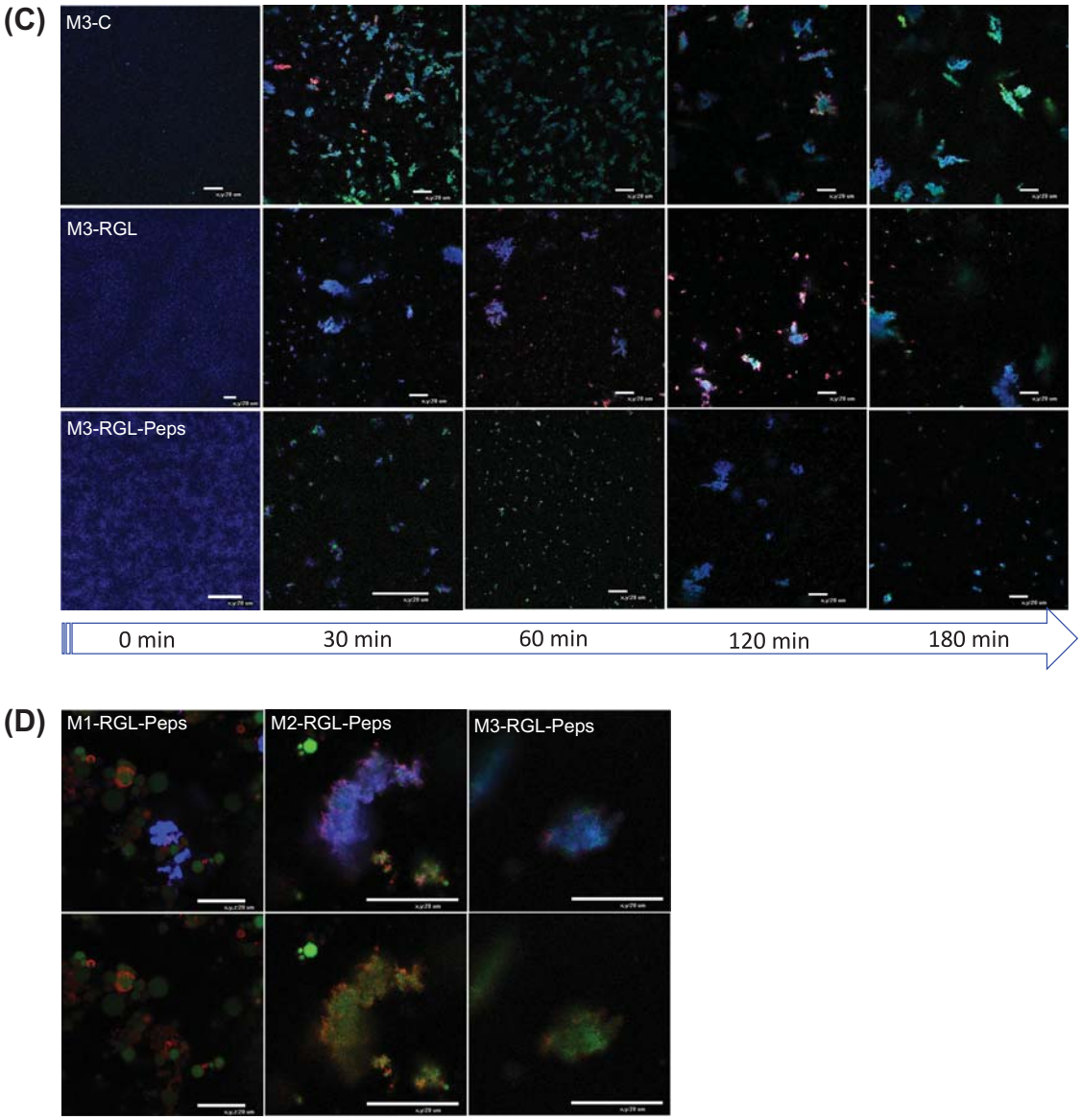



**Fig. 5** (*continued*)

than to matrix reorganization. It is well documented that fully protonated long chain fatty acids accumulates at the oil-water interface and inhibit the lipolysis reaction by gastric lipase (Gargouri et al., 1986; Pafumi et al., 2002). In our systems, the inhibition of *in vitro* lipolysis is delayed (M2 and M3), probably because the fatty acids initially liberated are short and medium chain fatty acids. These fatty acids are mainly deprotonated at the pH used for mimicking infant gastric conditions (Supplementary data Fig. 1), they are soluble in the aqueous phase and therefore do not accumulate at the interface. The lipolysis kinetics in M1 differed from those observed in M2 and M3. While the lipolysis rates were initially high in M2 and M3 and decreased after 60 min, the lipolysis rate in M1 was initially slow and increased only after 120 min. This is reminiscent of the hydrolysis of milk fat globules by pancreatic lipase, which becomes triggered upon lipolysis by the formed FFA or by the preliminary action of gastric lipase (Bernbäck et al., 1989; Gargouri et al., 1986). The decrease of pH to 4 after 120 min is also an important factor enhancing lipase activity.

Both the 'quantity' and the 'quality of interface' available for lipase adsorption are generally recognized as two key parameters that control the kinetics of catalysis in heterogeneous biphasic systems (Benzonana & Desnuelle, 1965; Golding et al., 2011; Lengsfeld et al., 2004; Verger & de Haas, 1976). However it is the first time it is clearly demonstrated that the 'quantity of interface' is the predominant parameter in gastric neonatal digestion of milk fat using gastric lipase and milk emulsions having the same chemical composition but differing structures. This finding is in accordance with the study of Golding et al. (2011) and in contrast with the study of Garcia et al. (2014), indicating that homogenization of milk which induces an increase in milk surface area available for lipase adsorption did not increase gastric lipolysis. The apparent contrast between our results and the conclusions of Garcia et al. (2014) may be explained by the fact that: (1) we present kinetic data whereas the other authors present final lipolysis extent only, (2) our comparison is done on matrices having exactly the same composition whereas they compare different batches of milk having close composition which may have reinforced variability. The

predominant effect of quantity of interface on gastric lipase activity is in line with the very strong tensioactive properties of this lipase. Gastric lipases, including HGL, RGL and dog gastric lipase, get inserted in phospholipid monolayers up to a critical pressure of 25 mN $m^{-1}$ (Bénarouche, Point, Parsiegla, Carrière, & Cavalier, 2013). Contrary to the other main digestive lipase, *i.e.* the pancreatic triglyceride lipase, which is very sensitive to the quality of interface and the presence of phospholipids (Lengsfeld et al., 2004), gastric lipase, here RGL, is able to attack at the same initial surface-weighted hydrolysis rate (0.1–0.2 μmol $min^{-1}$ $m^{-2}$) a trilayered interface such as the one of the milk fat globule membrane found in M1 and a neoformed interface based mainly on caseins and a single phospholipid and protein layer found in M2 and M3. This ability of RGL and by analogy of HGL to attack raw milk fat globules is in agreement with the fact that pre-hydrolysis of native milk fat globules by HGL favored their subsequent hydrolysis by pancreatic lipase (Bernbäck et al., 1989) Nevertheless, since the rate of M1 lipolysis is enhanced after 120 min of hydrolysis, and thus becomes higher than that of homogenized matrices in terms of surface-weighted hydrolysis rate (M1 = 7.4 *vs* M2 = 1.3 *vs* M3 = 3.4 μmol $min^{-1}$ $m^{-2}$), it seems that milk fat globule membrane and neoformed interfaces are recognized differently by gastric lipase upon generation of FFA and these latter also enhanced gastric lipase activity on milk fat globule membrane at low lipolysis levels. As M1 presents native milk fat droplets of similar size to mature human milk (Lopez & Ménard, 2011; Roman et al., 2007) whereas M2 and M3 present droplets size similar to infant formulas, the gastric lipolysis kinetics reported in this study are supposed representative of the ones reported respectively on human milk or infant formula *in vivo*. Little *in vivo* kinetic data is available. Roman et al. (2007) reported overall lipolysis of 4.7% in 30 min and 6.1 ± 2.6% in 180 min in gastric aspirate of preterm newborns ($N = 9$) after administration of infant formula enriched in medium chain triglycerides. The lipolysis kinetics observed in our *in vitro* model on M2 and M3 are in the same range and thus appear as physiologically relevant: hydrolysis degrees ranging between 4.6% and 3.6% at 30 min and reaching 7.2–13.5% in 180 min are observed. In human milk, Roman et al. (2007) reported more variability in lipolysis in relation with the limited number of newborns included in the study ($N = 2$). Overall lipolysis was lower than in infant formula at 30 min averaging 3.7% but higher by the end of the gastric phase reaching around 10.7%. The authors assumed that bile salt stimulated lipase active in raw maternal milk could explain this higher lipolysis level in human milk. It is important to note also that the human milk used in the study had a low fat content (2.05 ± 0.49 g/100 g TG) compared to the formula (3.45 ± 0.14 g/100 g TG) or to our model formula (3.27 ± 0.01%). In our model matrix M1 based on heat-treated (54 °C, 20 min) bovine milk, the bovine endogenous lipase has been inactivated and thus cannot contribute to the lipolysis. It will be interesting studying its contribution to the gastric lipolysis of human milk by comparing heat-treated *versus* raw human milk in a future study.

The main products of lipolysis obtained from the matrices are *sn*-1,2(2,3) DAG and FFA, these latter being mainly short and medium chain fatty acids. This is the expression of both a low hydrolysis level and the stereopreference of gastric lipase for the *sn*-3 position of TAG (Carrière et al., 1997). The rapid enrichment in short chain fatty acids in the FFA released from the two processed matrices M2 and M3 results from the specific regiodistribution of acyl moieties in cows' milk fat TAG (like in human milk): short and medium chain fatty acids are mainly in *sn*-3 position (87–92% of C4-C8 in cow's milk and 82% of C10 in human milk) whereas palmitic acid is predominantly in *sn*-2 position (72% in human milk and 42% in cows' milk) (Innis, 2011; Jensen et al., 1978). This selective enrichment is delayed in the case of M1 which is hydrolyzed at a slower rate. This suggests that TAG molecules accessible to gastric lipase at the oil-water interface might not be the same in M1 *vs* M2–M3 and that a segregation of TAG with longer acyl chains occurs in M1. This hypothesis is coherent with the complex crystallization behavior of milk fat TAG under the form of milk fat globules and with the reported segregation of TAG with preferential crystallization of high melting point fat at the periphery of the globule (Lopez et al., 2002). A preferential release of C6:0 to C12:0 acyl moieties from raw cream and pasteurized cream was also reported by Gallier et al. (2013), but the method of fatty acid analysis used by these authors did not allow butyric acid measurements. Garcia et al. (2014) also presented a *sn*-2 monoglycerides fraction profile with an enrichment in C16:0 (*sn*-2 preferential position) and C18:1 *cis 9*, and a decrease in C4:0 to C8:0 *sn*-3 located, which is coherent with our results. The specific release of short and medium chain fatty acids from cows' milk and of medium chain fatty acid from human milk constitutes a very efficient and rapid energy supply for the newborn (Lindquist & Hernell, 2010). These fatty acids are metabolized fast after passive but efficient absorption in the gastric compartment to the portal system. In addition, these medium to short chain fatty acids and corresponding partial glycerides provide protection against enteral pathogens (Hamosh et al., 1999). The relative decrease in C16:0 in the released FFA (10–20% of total fatty acids) compared to the quantity initially esterified in TAG (37%) is in line with its low *sn*-3 regiodistribution (13% in cow's milk and 8% in human milk). Such distribution favors its intestinal absorption as *sn*-2 MAG and limits loss under the form of calcium soaps (Innis, 2011).

### 4.2. Proteolysis of the matrices and its influence on emulsion destabilization

Milk stabilized emulsions are expected to undergo major changes in their particle size in adult gastric phase due to the effect of low pH, high ionic strength which modifies droplets charge (Singh, 2011). In our systems, the slow neonatal decrease of pH induced limited aggregation on M1 and M2 whereas the heat-treated matrix is more sensitive to acid aggregation in accordance with the higher pH at gelation reported for heat-treated milks (Lucey & Singh, 1997). The most destabilizing factor for the three emulsions was the hydrolysis of the adsorbed protein layer by pepsin. This finding is in agreement with previous study indicating that proteolysis induces a loss of charge on the droplets surface and a reduction of the adsorbed layer (Singh, 2011). The produced peptides that are maintained at the interface of the droplets do not provide sufficient electrostatic repulsions nor steric stabilization as the intact proteins. As a result the emulsion is highly susceptible to aggregation which is observed for all the matrices.

In agreement with previously published results, most of the whey and membrane proteins resisted gastric digestion but not caseins which were extensively hydrolyzed (Bouzerzour et al., 2012; Chatterton et al., 2004; Henderson, Hamosh, Armand, Mehta, & Hamosh, 1998). This high susceptibility to pepsinolysis was explained by the flexible random structure of caseins in solution. In agreement with Macierzanka et al. (2009), caseins proteolysis was accentuated in M2 and M3 when part of it was adsorbed at the oil-water interface. The 22 (in M1) to 49% (in M2) caseins hydrolysis reached after the first 60 min digestion accounted for 9–20% total protein hydrolysis which is in line with the 15% proteolysis reported in gastric aspirate of preterm infants at 50 min post meal ingestion by Henderson et al. (1998). This limited proteolysis is generally explained by low pepsin output and postprandial pH (4.0–6.0) higher than pepsin optimal pH (3.0). Functionally, this limited proteolysis enhances the protective functions of milk by preserving the bioactivity of specific milk proteins

(immunoglobulin A, lactoferrin, lysozyme) and allows the delivery of aggregated but preserved milk fat globules to the duodenum in the case of M1 matrix.

### 4.3. Potential impact of the structure of infant formulas on infant physiology and gastric emptying

Processing infant formulas modifies the mechanism of gastric aggregation of milk fat globules and kinetics of release of fatty acids and peptides. These two parameters could influence gastric emptying regulation in infants, pancreatic secretion and overall digestion and absorption. As described for adults, the release of amino/fatty acids in the upper duodenum stimulates cholecystokinin secretion which stimulates pancreatic and bile secretions and slows down gastric emptying (Hildebrand et al., 1998). Acid stability or instability of emulsions in the gastric phase also influence their gastric emptying in adults with delayed emptying of stable emulsions. Similar regulation is likely to occur in infants. So the fat ultrastructure modification resulting from infant formulas processing could play a part in the slower gastric emptying of infant formulas compared to unprocessed human milk having otherwise similar caloric content and casein/whey protein ratios.

Our study also indicates that the initial size of milk lipid droplets is increased during gastric phase but that emulsions with smaller droplets size compared to emulsions of larger droplets size remain with smaller droplets size in the duodenum which had already been noticed in adult gastric digestion (Armand et al., 1999. The complementary intestinal lipolysis could thus be accelerated for homogenized matrices though the total intestinal extent of lipolysis already high may not differ (Garcia et al., 2014). The acceleration of gastric lipolysis and possibly intestinal lipolysis of processed matrices may impact postprandial lipid metabolism (Michalski, 2009). Proofs of evidence of long term additional effects of early dietary lipid structure on fat accumulation and metabolism in animals were recently produced by Oosting et al. (2014).

### 4.4. Pros and cons of the simulated gastric in vitro digestion models

The lipolysis (<10% TAG hydrolysis) and proteolysis (9–20%) degrees obtained with the semi-dynamic model after 60 min gastric digestion were in agreement with the levels reported *in vivo* (Henderson et al., 1998; Roman et al., 2007). Many *in vitro* gastro-intestinal models present 60 min gastric phase. A shortening of the present model is acceptable to get an insight of the difference in hydrolysis kinetics between the three matrices. However it would lead to an underestimation of the lipolysis extent specifically for the minimally-processed matrix M1. A continuous decrease of pH, mimicking more closely physiological gastric acidification could have been adopted using for instance a dynamic digester (Ménard et al., 2014), but reduces the possibility of direct titration of FFA with pH-stat technique.

## 5. Conclusions

Our study provides evidence that minimally-processed model infant formulas based on dispersed native milk fat globules, which have bigger droplets and native interfaces, are lipolysed and proteolysed slower than model processed infant formulas during gastric digestion. It also indicates that the differences in initial structure is maintained during the gastric phase of digestion. Our findings show that among all the structural parameters, the specific surface area of the droplets in the infant formula is the key parameter to control the rate of gastric lipolysis, the overall degree of gastric lipolysis and also the pattern of released fatty acids. This parameter also indirectly controls proteolysis, since adsorbed proteins are hydrolyzed much faster than solubilized proteins. These differences in luminal gastric digestion may have important physiological implications in infants. These differences were observed using an original semi-dynamic *in vitro* model of gastric neonatal digestion based on and validated against *in vivo* data. The next step in our work will be to apply the new model to human milk to measure the additional effect of the endogenous lipase and bile salt stimulated lipase in maternal milk on gastric neonatal digestion. The results of this study will be useful to food scientists and infant food manufacturers as they provide evidence for the direct influence of the structure of the emulsion in neonatal gastric digestion. We suggest manufacturers of infant food should focus on developing soft processes with minimal impact on milk emulsion structure in the near future as a priority.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.03.001.

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