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ABSTRACT

Donor human milk, pasteurised for safety reasons, is the first alternative for feeding preterm infants when mothers' own milk is unavailable. Breastmilk pasteurisation impact on lipid digestion and absorption was evaluated by a static *in vitro* digestion model for preterm infants coupled with intestinal absorption using Caco-2/TC7 cells. Lipid absorption was quantified by digital image analysis of lipid droplets, by measurement of basolateral triglyceride concentration and by analysing the expression of major genes involved. After *in vitro* digestion, lipolysis extent was 13% lower in pasteurised human milk (PHM) than in raw human milk (RHM). In Caco-2/TC7 cells, the number of lipid droplets was identical for both milk types, while the mean droplet area was 17% smaller with PHM. Altogether, pasteurisation decreased the pre-lipolysis of human milk. This initial difference in free fatty acid amount was only partially buffered by the subsequent processes of *in vitro* digestion and cellular lipid absorption.

1. Introduction

Preterm birth, defined as any birth before 37 completed weeks of gestation, accounted for 7.3% of all births in France in 2015 (Euro-Peristat Project. European Perinatal Health Report, 2018). It is associated with increased neonatal morbidity and long-term neurodevelopmental impairment (Ancel et al., 2015; Pierrat et al., 2017). Indeed, preterm infants may be exposed to several complications such as necrotising enterocolitis, retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular haemorrhage, late-onset sepsis and can also suffer from cognitive developmental delay (Ancel et al., 2015).

Many studies have reported that human milk could reduce complications due to prematurity. Indeed it decreases risk of necrotising enterocolitis, retinopathy of prematurity and late-onset sepsis and improves growth and neurodevelopment (Gephart et al., 2017; Menon & Williams, 2013). For instance a long-term study on American children has shown an association between the amount of human milk received during infants' hospitalisation and cognitive development score (Vohr et al., 2007). Therefore human milk is the gold standard in neonatal nutrition, especially for preterm infants (Gephart et al., 2017). However mothers who delivered prematurely often have difficulty to breastfeed their infant (Bonet et al., 2011). According to World Health

Organization (2011), donor human milk (DHM) is the recommended alternative for feeding preterm infants when mothers' own milk is unavailable.

Effectively DHM from a human milk bank is a valuable resource for premature infants whose mothers are unable to provide an adequate supply of milk. However, the use of DHM presents significant challenges including the need for pasteurisation. Holder pasteurisation (62.5 °C for 30 min) is recommended by all international human milk bank guidelines to prevent infections potentially transmitted by DHM (AAP Committee on Nutrition et al., 2017; Moro et al., 2019). Nevertheless, pasteurisation modifies some of milk properties, as protein denaturation and aggregation on the milk fat globule. Pasteurisation notably denatures nutritive and bioactive components such as bile salt stimulated lipase (BSSL) (Henderson, Fay, & Hamosh, 1998; Picaud & Buffin, 2017) that is essential for the efficient digestive lipolysis of milk triglycerides by the premature infant (Lindquist & Hernell, 2010). Thus, intestinal lipid absorption could be reduced with pasteurised as compared to raw milk (Andersson, Sävman, Bläckberg, & Hernell, 2007), but the impact on growth is still widely debated (Brownell et al., 2018; Madore et al., 2017). Indeed, BSSL improves lipid digestion and absorption through its ability to hydrolyse a broad variety of lipids but this lipase has been said to contribute to only 20–25% of fat absorption in premature infants (Picaud et al., 2018).

Other mechanisms are involved in intestinal lipid absorption and must be explored. Even if several *in vitro* studies reported an impact of pasteurisation on digestive lipolysis (de Oliveira et al., 2016), none explored the subsequent impact on enterocyte lipid absorption.

The aim of our study was thus to determine the impact of human milk pasteurisation on preterm infant gastrointestinal digestion using an *in vitro* static digestion model and to further evaluate its effect on lipid absorption using Caco-2/TC7 cells as an intestinal fatty acid (FA) absorption model.

2. Methods

2.1. Human milk sample preparation and characterisation

Mature human milk samples from two donors were obtained from a regional human milk bank at the University Hospital Centre in Lyon (France). Donors gave their informed written consent. Lactating women collected their milk between 5 and 9 months after term delivery and stored it at −20 °C.

Milk was thawed at 4 °C and equal volumes of milk from each donor were pooled. Half of the pool, called raw human milk (RHM), went back to storage at −20 °C until digestion. The other half underwent Holder Pasteurisation (62.5 °C, 30 min) before going back to storage at −20 °C. The latter pool was called pasteurised human milk (PHM).

Because BSSL is known to be inactivated by pasteurisation (Henderson et al., 1998), BSSL activity was measured in quadruplicate in RHM and in PHM as assessed by pH-stat *(Titrando 842, Metrohm SA, Courtaboeuf, France)* and using tributyrin as substrate at pH = 8 (as detailed by Minekus et al. in the Supplementary Materials section (Minekus, Alminger, Alvito, Ballance, & Bohn, 2014)).

Macronutrient composition of RHM and PHM was assessed by infrared spectrophotometry, using a MIRIS Human Milk Analyzer 2 *(Miris AB, Uppsala, Sweden)* and calibration adjustment was applied (Buffin et al., 2017a; Casadio et al., 2010). Total FA were quantified in PHM and RHM by gas chromatography coupled to a flame ionization detector *(GC7890A, Agilent Technologies, Les Ulis, France)* by direct transmethylation in the presence of internal standard (C13:0) without prior extraction, as described previously (López-López, López-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargalló, 2002).

2.1.1. In vitro digestion

Six mL of RHM and PHM were submitted to gastrointestinal digestions using a static *in vitro* model (Bourlieu et al., 2014; Ménard et al.,

2018) adapted for simulating preterm infant digestion. The amount of gastric and intestinal enzymes were adjusted according to the mean body weight, which was considered as 2 kg for preterm infant in order to simulate a one-month preterm infant. The gastrointestinal digestion was performed in triplicate for each pool of milk. Two consecutive one-hour steps were conducted in a water bath at 37 °C under stirring: a gastric phase followed by an intestinal phase. For the gastric phase, pH was set up at 5.3 with 1 M HCl. Pepsin (63 U/ml of gastric content/kg of body weight of infant) and gastric lipase (4.5 U/ml of gastric content/kg of body weight of infant) were added as rabbit gastric extract. Simulated gastric fluid was composed of 94 mM NaCl and 13 mM KCl. The total volume of the gastric phase was composed of 63% of meal and 37% of gastric secretion.

After 60 min of gastric digestion, pH was increased to 7 by addition of 1 M NaOH in order to inhibit gastric enzymes and then adjusted to the intestinal pH of 6.6 using HCl. For the intestinal phase, porcine pancreatin (59 U of pancreatic lipase/ml of intestinal content) and bovine bile (1.6 mM bile salts) were added. Simulated intestinal fluid was composed of 164 mM NaCl, 10 mM KCl and 85 mM NaHCO$_3$. Just before the intestinal phase, CaCl$_2$ was added to reach 3 mM. The total volume of the intestinal phase was composed of 39% of meal, 23% of gastric secretion and 38% of intestinal secretion.

Aliquots were collected before digestion (G0), after 60 min of gastric phase (G60), and after 15, 30 and 60 min of intestinal phase (I15, I30 and I60, respectively). Samples for lipid analysis were immediately submitted to lipid extraction before storage at −20 °C for thin layer chromatography and free fatty acids (FFA) analyses. Aliquots collected from the intestinal compartment at 60 min were frozen at −80 °C for lipid absorption analyses.

2.1.2. Lipolysis characterisation

Thin layer chromatography was performed to follow the lipolysis. After direct cold lipid extraction, adapted from Folch method, samples were loaded on silica plates in order to evaluate the appearance of lipolysis products and the disappearance of triacylglycerols (TAG) as described previously (Ménard et al., 2018).

Image analysis, measuring the densitometry of the grey intensity of bands visible on plates, were performed using the software Image Quant TL™ *(GE Healthcare Europe183 GbmH, Velizy-Villacoublay, France)*. This allowed a semi-quantitative analysis of the digesta.

FFA were also analysed and quantified by gas chromatography coupled to a flame ionization detector. After lipid extraction in the presence of internal standards (C5:0, C11:0, C17:0), the FFA were purified by a solid phase extraction using strata-NH2 (1 ml/100 mg) SPE from Phenomenex. Briefly, the chloroform phase containing FFA was adsorbed on Strata-NH2 after stabilization of the sorbent by heptane. Elution of FFA was performed by a mix of diethyl ether/formic acid 2%. Then, 300 µl of FFA was methylated with 1 ml of BF3-methanol *(Sigma,Saint-Quentin-Fallavier, France)* for 10 min at 70 °C. After a cool down on ice, the solution was washed by 1 ml of K2CO3 10% in water and FFA Methyl Ester were extracted by 0.5 ml of hexane after centrifugation. FFA Methyl Ester, resulting from the digestion process, were identified by comparing the retention time with a standard mix (mixture ME 100) from Larodan *(Larodan AB, Solna, Sweden)*. Quantification was done by an external calibration and internal standard correction.

The lipolysis extent was expressed at a given time as the percentage level of FFA (in moles) released before or during *in vitro* digestion versus the total FA present in the pasteurised or raw undigested milk:

$$Lipolysis\ extent_{G0} = 100 * \frac{FFA_{G0}\ (moles)}{TotalFA_{undigested\ milk}\ (moles)}$$

$$Lipolysis\ extent_{I60} = 100 * \frac{FFA_{I60}\ (moles)}{TotalFA_{undigested\ milk}\ (moles)}$$

We also calculated the digestive lipolysis extent between the

beginning and the end of *in vitro* digestion as the percentage of additional FFA released between the beginning (G0) and the end (I60) of *in vitro* digestion, versus total FA present in the undigested milk minus FFA already present at G0:

$$Lipolysis\ extent_{I60-G0} = 100 * \frac{FFA_{I60}(moles) - FFA_{G0}(moles)}{TotalFA_{undigested\ milk}(moles) - FFA_{G0}(moles)}$$

2.2. Caco-2/TC7 cell culture

Caco-2/TC7 intestinal cells, kindly provided by Dr M. Rousset (InsermU505, Paris, France), are derived from a human colon adenocarcinoma. They are able to undergo spontaneous differentiation that leads to a cell monolayer formation expressing several morphological and functional characteristics of enterocytes (Chantret et al., 1994; Sambuy et al., 2005). Among Caco-2 cells, the TC7 clone has been described to be the most relevant for FA absorption studies (Salvini, Charbonnier, Defoort, Alquier, & Lairon, 2002; Vors et al., 2012).

Cells were cultured on 75 cm^2 flasks in high-glucose (4.5 g/L) medium with glutamine *(DMEM GlutaMAX; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA)* supplemented with 20% foetal calf serum, 1% of non-essential amino acids and 1% penicillin/streptomycin *(Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA)*. Cells were grown in 37 °C humidified atmosphere 90% air and 10% CO$_2$ and were used between passages 41 and 44.

For experiments, cells were split with 0.05% Trypsin-EDTA when 80% of confluence was reached and were seeded on permeable Transwell® filter inserts at a density of 5 × 10^4 cells/cm^2 *(24 mm insert, 0.4 μm pore size Polyester membrane, Corning Costar, Cambridge, MA, USA)* in 6-well plates to reproduce the intestinal barrier. This device enabled the establishment of an apical compartment (upper one) mimicking the intestinal lumen and a basolateral compartment (lower one) mimicking the portal and lymphatic circulation.

Cells were grown to confluence in complete medium for one week. Cells were then cultured in asymmetric conditions, with 2 ml medium containing foetal calf serum in the lower compartment and 1.5 ml serum-free medium in the upper compartment until total differentiation (around 21 days after seeding). The medium was changed every 2–3 days. Preliminary experiments revealed that the TAG secretion in the basolateral compartment increased until 16 h after incubation of Caco-2/TC7 cells with lipids, with no further significant increase from 16 h to 24 h. Various digested milk dilutions were tested (1/10th to 1/80th) in order to find the dilution factors that ensure both an absence of toxicity on the Caco-2 cells and a measurable chylomicron secretion. Therefore, Caco-2/TC7 cells were subsequently incubated during 16 h with 1.5 ml of 1/20th diluted PHM- vs RHM-digestion media.

Cell monolayer integrity was assessed by measuring the transepithelial electrical resistance (using a *Millicell ERS-2 apparatus, Millipore, USA*) and occludin immunostaining (*dilution 1:200, Santa Cruz Biotechnology, CA, USA*).

2.2.1. Lipid uptake analysis

Intracellular lipid droplets were stained by Oil Red O *(Sigma, Saint-Quentin-Fallavier, France)* and were quantified after acquiring 4 images per well (9 wells per milk type: triplicate incubations of the 3 different digestions) by standard light microscopy. Using ImageJ software *(U.S. National Institutes of Health, Bethesda, Maryland, USA)*, the contrast was enhanced by 3% in order to better distinguish smallest droplets. Quantification was then performed by selecting and counting pixels with a saturation larger than 140 with the Otsu thresholding method. Segmentation was then applied automatically using the Watershed method of ImageJ in order to count and measure lipid droplets individually. This protocol was adapted from Deutsch et al. (Deutsch, Schriever, Roscher, & Ensenauer, 2014).

Triglyceride secretion was measured in basolateral medium using a Fluorometric Triglyceride Assay Kit according to the manufacturer's instruction *(Abcam, Cambridge, United Kingdom)*.

2.2.2. RNA extraction and real-time quantitative PCR

Total RNA was extracted from cells with Tri Reagent *(Sigma, Saint-Quentin-Fallavier, France)* and suspended in RNase-free water. RNA concentration was measured with Multiskan GO microplate spectrophotometer *(Thermo Fisher Scientific, Waltham, MA, USA)* and RNA samples with A260/280 ratio between 1.7 and 2.1 were considered of good purity. Reverse transcription was performed using PrimeScript RT reagent kit *(Ozyme, Saint Quentin en Yvellines, France)* with one microgram of RNA. Real-time PCR assays were performed using a Rotor-Gene Q *(Qiagen, Hilden, Germany)* and SYBR qPCR Premix Ex Taq (Tli RNaseH Plus) reagents. PCR primers are listed in Supplementary Table 1. Results were normalised by using TBP (TATA box binding protein) mRNA concentration, measured as reference gene in each sample.

2.3. Statistical analysis

Statistical analyses were performed using R software version 3.5.2. Welch's unequal variances two-sided *t*-test was performed to analyse lipolysis extent. Two series of cell cultures were realised in triplicate with the three digestions of RHM and PHM. To avoid the pseudo-replication fallacy, mixed linear models were applied to analyse the responses of interest: total number of pixels in droplets per image, total number of droplets per image, mean area of droplets, triglyceride concentration in basolateral medium and expression of some genes involved in lipid metabolism. Each response was predicted from the type of milk (fixed effect factor), the series of cell culture (random effect factor) and the digestion assay (random effect factor, crossed with the series of cell culture and the type of milk) with a linear mixed effect model, fitted with the lme function of the nlme package, using treatment contrasts. When technical replicates were performed for each well, the well (random effect factor, nested into the digestion factor) was also included in the predictors. To assess the effect of pasteurisation on the response, an F-test conceptually similar to the F-test of an ANOVA was computed for the milk type factor.

For each family of tests (*in vitro* lipolysis, Oil Red O staining, gene expression analysis), we reported both the raw p-values and the adjusted p-values accounting for multiple testing using Benjamini-Hochberg procedure (false discovery rate (FDR) correction). All results are reported as mean ± standard error of the mean (SEM).

3. Results

3.1. Human milk composition

PHM and RHM had the same macronutrient composition, which consisted of 23.0 ± 2.0 g/L of fat, 14.5 ± 0.5 g/L of proteins and 70.5 ± 0.5 g/L of carbohydrates.

Total FA analysis (i.e. both esterified in TAG and phospholipid and as Non-Esterified FA) by gas chromatography confirmed no difference between PHM and RHM (22.77 ± 0.09 vs 21.35 ± 0.75 g/L respectively; p = 0.20). Oleic and palmitic acids were the most abundant FA for both milks, followed by linoleic and myristic acids (Supplementary Fig. 1).

3.2. Bile salt stimulated lipase activity

BSSL activity was 34.5 ± 3.7 U/ml in RHM and 4.8 ± 1.6 U/ml in PHM. It means that pasteurisation resulted in 86.1% loss of lipase activity (p = 0.007).
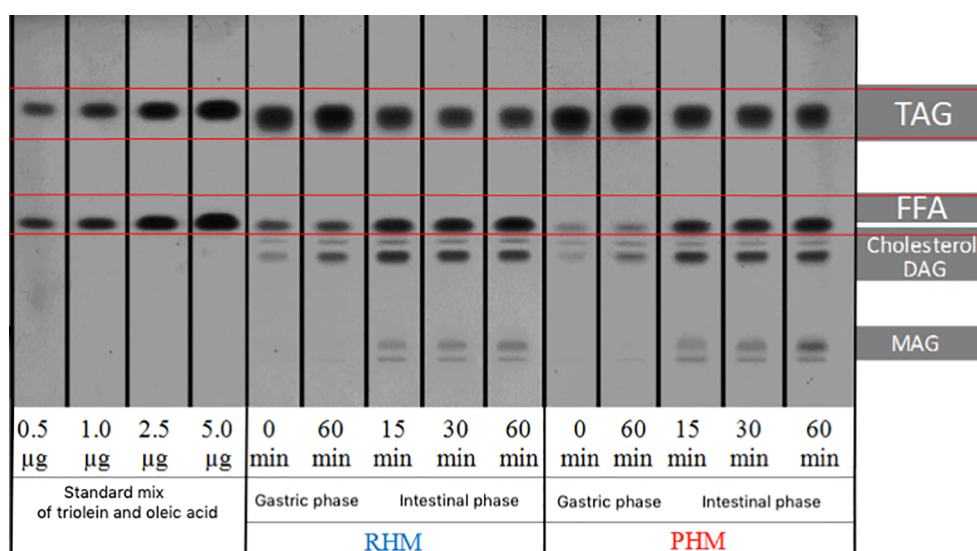
Fig. 1. Thin layer chromatography plate of the first *in vitro* digestion of raw human milk (RHM) versus pasteurised human milk (PHM) showing lipid classes including triacylglycerols (TAG), free fatty acids (FFA), cholesterol, diacylglycerols (DAG) and monoacylglycerols (MAG).

3.3. Digestive lipolysis kinetics in vitro

Lipolysis kinetics of PHM and RHM are shown in Figs. 1 and 2. Lipolysis occurred mainly in the intestinal phase. During digestion, TAG were hydrolysed into FFA, diacylglycerol and monoacylglycerol. However, by gas chromatography coupled to a flame ionization detector, lipolysis extent was 52% lower in PHM versus RHM at G0 (before *in vitro* digestion) (6.1 ± 0.6% and 12.7 ± 0.2% respectively; p = 0.006 and FDR-adjusted p = 0.017) and was at the limit of the statistically significant level at I60 (at the end of the gastro-intestinal digestion) (39.3 ± 0.9% versus 45.4 ± 1.7%; p = 0.052 and FDR-adjusted p = 0.079). This 13% difference at I60 was mainly due to the difference at G0. Indeed, we did not observe any difference in the extent of lipolysis that occurred between G0 and I60 for PHM and RHM (35.4 ± 1.4% versus 37.5 ± 1.9% respectively; p = 0.417 and FDR-adjusted p = 0.417) (Fig. 2). The particle size distribution of RHM and PHM before milk digestion (G0), at G60 and I60 was analysed (Supplementary Fig. 2). At I60, the same size of mixed compounds was found in both conditions (D [4;3] = 13,4 μm).

3.4. Cell monolayer integrity

Caco-2 cells were incubated during 16 h with digested milk, i.e. milk at the end of 60 min of intestinal digestion. The 1/20th dilution showed the highest triglyceride secretion (not shown) without being toxic for Caco-2 cells. Cell monolayer integrity assessed by transepithelial electrical resistance (not shown) and occludin immunostaining were unaffected. Cell monolayer was homogeneous and tight junctions remained intact (Supplementary Fig. 3).

3.5. Intestinal uptake of lipids

Caco-2 cells were incubated with the same amount of digestion media from both PHM and RHM. After 16 h of incubation with digested milk, intracellular lipid droplets were visible using Oil Red O staining (Fig. 3).

The number of droplets per image was identical for both milk types $(F_{(1,4)} = 1.75; p = 0.256$ and FDR-adjusted p = 0.256), while the mean droplet area was 17% smaller in the pasteurised condition (F
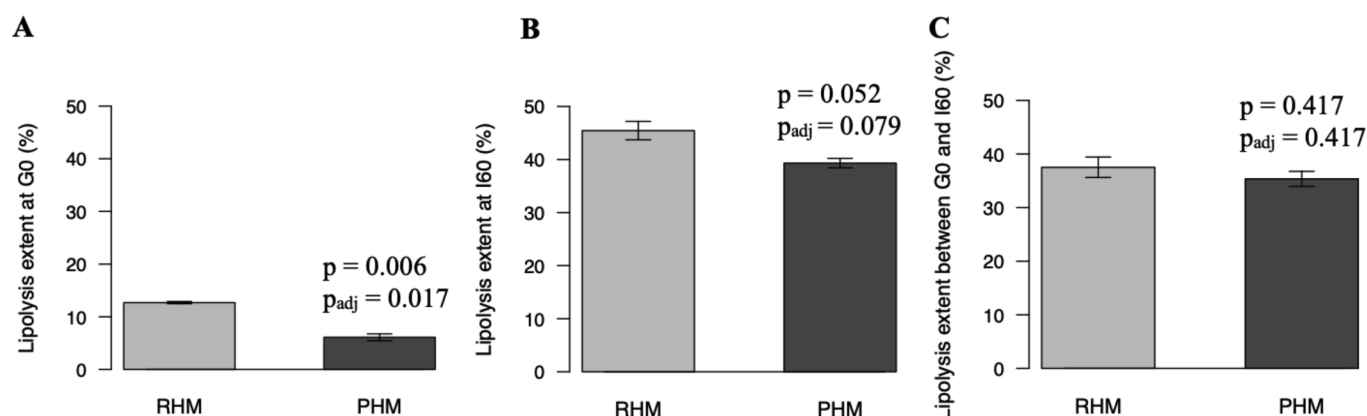

Fig. 2. Lipolysis extent (% of fatty acid released from total initially esterified fatty acid) in raw human milk (RHM) versus pasteurised human milk (PHM) before and during *in vitro* digestions: before the gastric phase G0 (A), at the end of the intestinal phase I60 (B) and during *in vitro* digestion between G0 and I60 (C). Lipolysis extent was determined by thin layer chromatography coupled to a flame ionization detector. Data are means ± SEM of triplicate analyses. p: raw value; p_{adj}: FDR-adjusted p-value for the 3 tests.
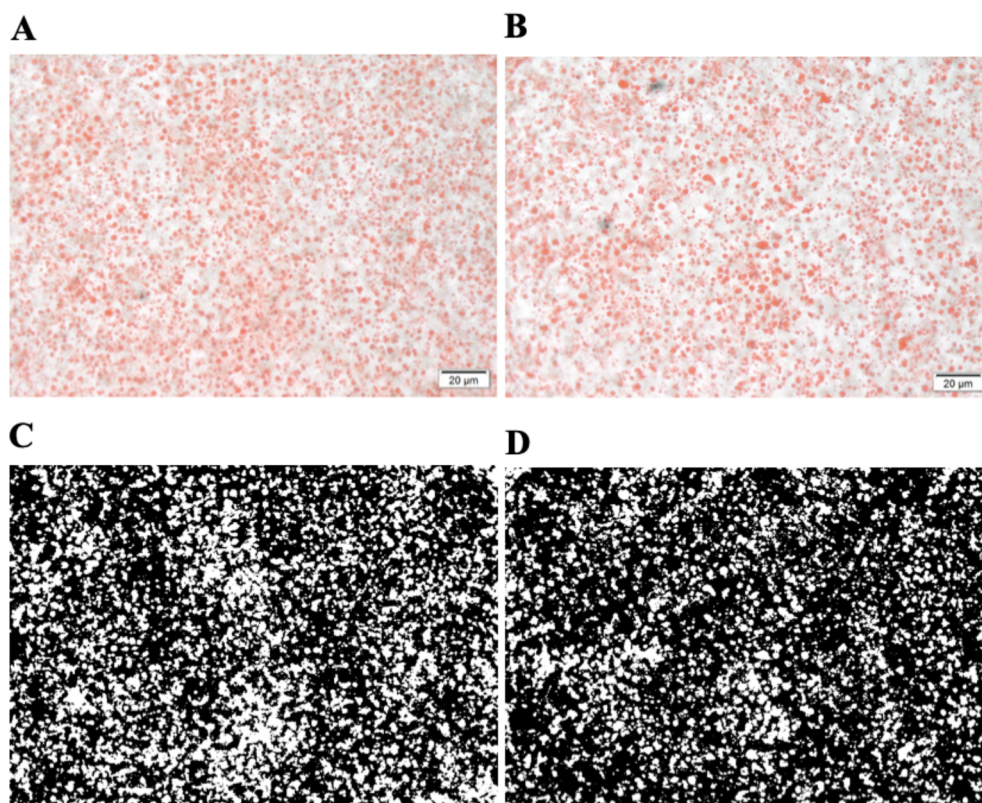
Fig. 3. Lipid droplets visualised after Oil Red O staining within cells incubated with raw human milk (RHM) (A) or pasteurised human milk (PHM) (B) and after digital image processing for RHM (C) and PHM (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(1,4) = 21.08; p = 0.010 and FDR-adjusted p = 0.040) (Fig. 4). There were 13% fewer pixels of lipid droplets in the pasteurised condition (F (1,4) = 9.72; p = 0.036 and FDR-adjusted p = 0.071). By contrast, the median droplet area was identical in both conditions (F(1,4) = 2.09; p = 0.222 and FDR-adjusted p = 0.256). Inspection of lipid droplet area distributions show that the difference in means was due to the presence of much smaller droplets in cells incubated with PHM whereas the number of very large droplets was similar in the both conditions.

Triglyceride secretion measured in the basolateral medium of Caco-2/TC7 cells was low when cells were incubated with medium alone devoid of lipids (0.79 ± 0.02 µmol/L, n = 3) and increased after 16 h incubation with digested human milk without significant statistical difference between PHM and RHM (36.1 ± 3.9 vs 42.6 ± 3.6 µmol/L respectively; p = 0.26) (Fig. 5).

3.6. Gene expression

After 16 h incubation of Caco-2/TC7 cells with digested milks, the expression of some genes involved in fatty acid transport (SLC27A4, FABP2), intracellular lipid re-synthesis (MOGAT2, DGAT2), lipid droplet (PLIN2) or chylomicron (MTTP, APOA4) formation was increased at least 2-fold compared to cells incubated in medium alone (Fig. 6). Among the studied genes, no significant gene expression difference between incubation with PHM and RHM was observed. Of note, the p-values obtained for PLIN2 and APOA4 genes were 0.036 and 0.031 respectively, but did not remain statistically significant after FDR correction considering that 13 genes related to lipid absorption were tested.

4. Discussion

The present experiments demonstrated that pasteurisation significantly decreased the pre-lipolysis during storage and processing of human milk and therefore decreased the endogenous release of FA before *in vitro* digestion, without qualitatively altering total fatty acid profile, but that difference was not amplified during *in vitro* gastrointestinal digestion. The difference in FA content was at the limit of statistical significance at the end of the gastrointestinal digestion, with 13% less FA in PHM than in RHM. Lipid uptake by Caco-2/TC7 cells was also 13% lower in PHM, and was associated with smaller intracellular lipid droplets, when incubated with digested PHM than with digested RHM. This suggests that the initial difference in FA content is not completely buffered by cellular uptake processes either. However, final TAG secretion in the basolateral medium of Caco-2/TC7 cells was not significantly impacted by pasteurisation.

A randomised controlled trial by Andersson et al. (2007) found a lower coefficient of fat absorption from pasteurised as compared to raw own mother's milk but the difference was not statistically significant. Moreover, they did not observe any significant difference in terms of weight gain between PHM and RHM. However, other studies show that raw human milk improves weight gain compared with pasteurised DHM in preterm infants (Brownell et al., 2018; Madore et al., 2017). These discrepancies can be partly explained by a loss of proteins and lipids by nasogastric tube during feeding, especially with pasteurised human milk. Such phenomenon has been attributed to increased adherence of the disrupted milk fat globules to syringe and tube (Vieira, Soares, Pimenta, Abranches, & Moreira, 2011). Discrepancies in *in vivo* results can thus be due to different techniques of milk administration (directly or via a plastic syringe/tube).

Most clinical studies compared raw own mothers' milk to pasteurised donor milk and differences in outcome could be related to higher nutrient content in raw (premature) milk when compared to donor (mature) milk (Schanler, 1980). A randomised clinical trial comparing raw and pasteurised own mothers milk did not find any difference in growth (Cossey et al., 2013) confirming the results from Andersson et al. (2007).

In our study, pasteurisation did not impact milk total lipid content. A review of the effects of human milk pasteurisation on nutrients published in 2016 reported various results depending on studies, heat-treatment process and analysis techniques (Peila et al., 2016). As in our
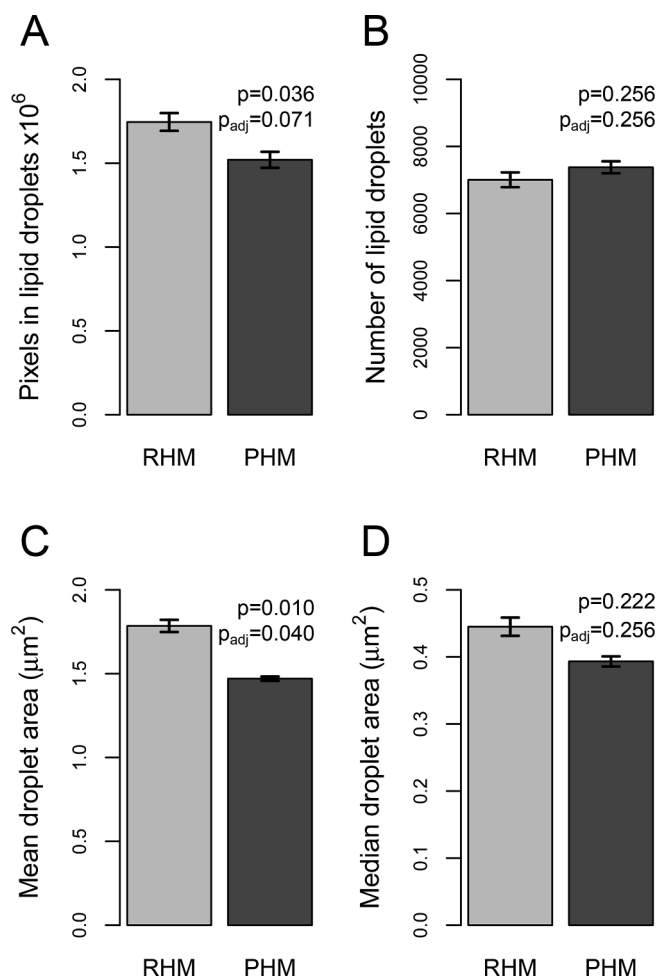
Fig. 4. Number of pixels in lipid droplets (A), number of lipid droplets (B), mean droplet area (C) and median of the droplet area (D) in Caco-2/TC7 cells after incubation with digested raw human milk (RHM) or digested pasteurised human milk (PHM), oil red O staining and digital image processing. Data are mean ± SEM. p: raw value; p_{adj}: FDR-adjusted p-value for the 4 tests.
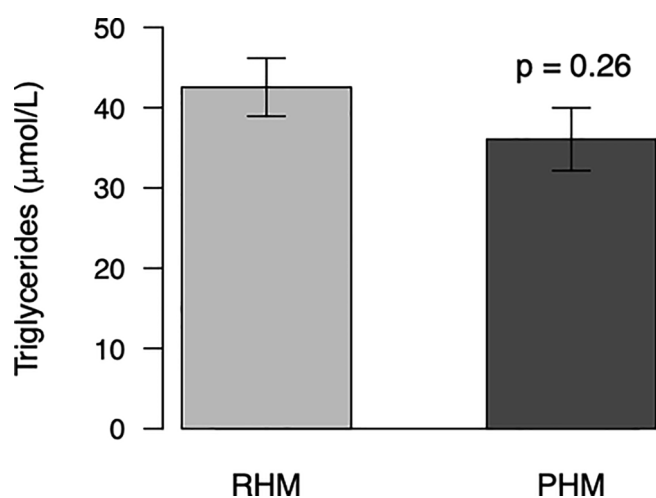

Fig. 5. Raw human milk (RHM) versus pasteurised human milk (PHM)-triglyceride secretion in basolateral medium of Caco-2/TC7 cells. Data are means ± SEM.

study, most studies found that total lipid content is preserved after Holder pasteurisation, as well as its FA composition (Peila et al., 2016; Picaud & Buffin, 2017). In contrast, a recent study by Piemontese et al.

with a high number of analysed milk samples demonstrated that Holder pasteurisation significantly modifies the macronutrient content of DHM, reducing the content of lipids by 5% and proteins by 2% (Piemontese et al., 2019). These differences may be explained by adherence of the disrupted milk fat globules to the container walls, if heat-treatment and feeding processes differ by the number of container changes. Moreover, samples submitted to thermal treatment in laboratories often consist of a few millilitres of milk, while human milk banks routinely use industrial pasteurisers, specifically designed and validated for the pasteurisation of larger quantities of human milk. In such real conditions, higher loss of human milk components is expected to occur due to the longer time needed to reach the expected temperature in the centre of the container (Picaud & Buffin, 2017).

Moreover, the impact of pasteurisation on inhibiting BSSL activity has been previously described (Picaud & Buffin, 2017), but in the present study we measured a low residual lipase activity in PHM. These discrepancies could be explained by several factors besides the volume of human milk processed, such as the time needed to reach the desired temperature and the time for cooling. A prolonged exposition of milk to high temperature could increase the alteration of milk properties (Buffin et al., 2017b). In our study, we tried to reproduce human milk bank recommended conditions with a short time to reach 62.5 °C and a fast time for cooling. This well-conducted pasteurisation could partly explain the persistence of a low residual BSSL activity.

Regarding lipid profile, oleic and palmitic acids were the most abundant FA in both PHM and RHM in our study, which is in accordance with recent literature (Thakkar et al., 2019).

This static *in vitro* digestion model aimed at mimicking as close as possible the digestive conditions of full-term infants (Bourlieu et al., 2014; Ménard et al., 2018). In our study, this model was adapted to simulate premature infant digestion ability. Lipolysis kinetics of human milk was also studied in preterm conditions using a dynamic simulator. A tendency to undergo a lower lipolysis for PHM than RHM in the intestinal phase was also observed (de Oliveira et al., 2016). Similarly, in our study, FFA tended to be lower in PHM than in RHM after *in vitro* digestion, but this effect was due to lower pre-lipolysis for PHM rather than to a difference occurring during the *in vitro* digestion itself. This initial difference in the amount of free fatty acids seemed to persist throughout the subsequent processes of *in vitro* digestion and impacted intestinal uptake.

Regarding cellular mechanisms of lipid uptake, FA uptake is mediated through passive diffusion and protein-facilitated processes. Because of their hydrophobicity, TAG do not cross the cell membrane. TAG absorption involves their hydrolysis to FA and monoacylglycerol in the intestinal lumen, lipid uptake by the enterocytes and resynthesis of TAG. Then TAG are channelled either towards cytosolic lipid droplets or towards chylomicrons for lymphatic secretion (Yen, Nelson, & Yen, 2015). The genes studied were chosen to represent all major steps in these processes. After incubation of Caco-2/TC7 cells with digested milks, expression of several genes was enhanced compared to cells incubated in medium alone. However, there were no overall difference in gene expression between PHM and RHM, although PLIN2 and APOA4 had significantly decreased expression in PHM before correction for multiple testing. How the lower mean droplet area with PHM is mechanistically associated with the apparent lower gene expression of these proteins located at the surface of both intracellular lipid droplets and chylomicrons will deserve to be elucidated. As supported by a recent review (Ko, Qu, Black, & Tso, 2020), PLIN2 is required for the formation of intestinal cytosolic lipid droplets while APOA4 facilitates the packaging of additional lipids into the core thereby promoting the production of larger-sized chylomicrons.

To advance the state of the art and evaluate the impact of *in vitro* milk digestive media on intestinal lipid uptake and absorption, Caco-2 cell line was used as an intestinal absorption model. Although this model is criticised because of its limited secretion capacity remaining a potential drawback for direct comparisons with *in vivo* physiological
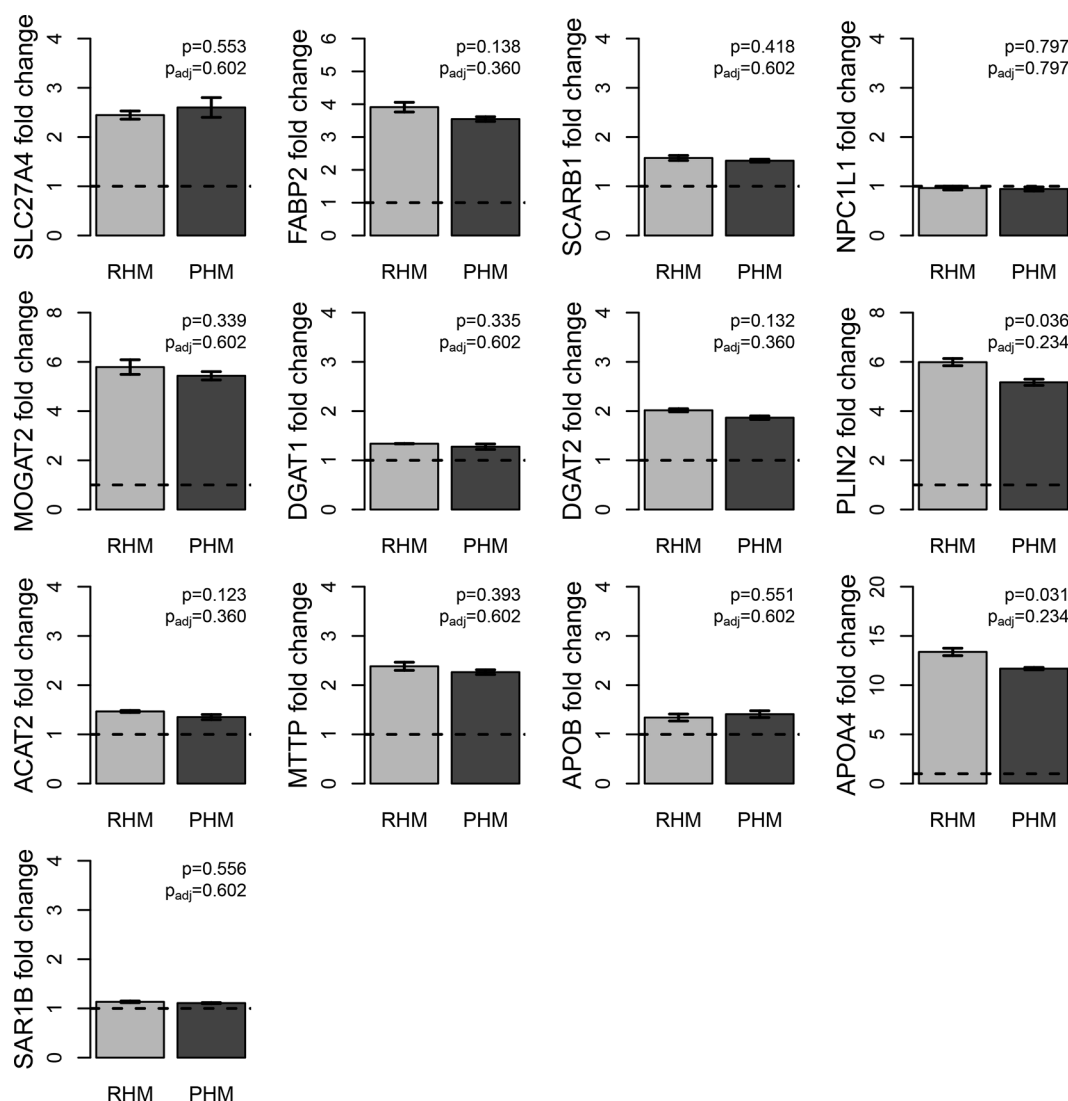
Fig. 6. Expression of genes involved in fatty acid transport (SLC27A4, FABP2), cholesterol transport (SCARB1, NPC1L1), lipid metabolism (MOGAT2, DGAT1, DGAT2, PLIN2, ACAT2), and chylomicron formation (MTP, APOB, APOA4, SAR1B) after incubation with RHM or PHM in Caco-2/TC7 cells. Dotted lines represent a fold-change similar to the medium alone. Data are expressed as fold-change versus medium condition devoid of lipids and represent means ± SEM. p: raw value; p_{adj}: FDR-adjusted p-value for the 13 tests.

state (Levy, Mehran, & Seidman, 1995), it provides an excellent *in vitro* model for investigation of intestinal lipoprotein metabolism and has been extensively used over the last thirty years (Sambuy et al., 2005). The Caco-2/TC7 clone has been chosen for its best capacity to absorb lipids compared to parental Caco-2 clone (Salvini et al., 2002). However, triglyceride secretion in Caco-2 cells is low which was attributed to low monoacylglycerol acyltransferase activity and MOGAT2 mRNA expression (Levy et al., 1995; Pauquai et al., 2006). However, in our culture conditions and in this TC7 clone of Caco-2 cells, MOGAT2 expression was clearly increased by incubation with either milk. In some studies, incubation of other Caco-2 clones with model mixed lipid micelles had no effect on MOGAT2 mRNA level (Pauquai et al., 2006). These discrepancies can be due to either differences across Caco-2 clones or to the use of different lipid sources. Indeed, partially digested milk fat globules could be more prone to activate the lipid absorption pathway in this cell model compared to artificial lipid micelles.

Apart from the persistence of a weak activity of the BSSL, our study had several limitations. First of all, a combination of enzymes has been used to mimic digestion but some intestinal enzymes were missing (phospholipases and brush border enzymes). In addition, we tried to limit the steps of freezing and thawing milk since these steps can also

have an impact on lipids (Vieira et al., 2011). To limit this bias, the same number of steps has been achieved for both milks.

The present *in vitro* results suggest that pasteurisation decreased the pre-lipolysis of human milk as compared to raw milk. This initial difference in the amount of free fatty acids seemed to persist throughout the subsequent processes of *in vitro* digestion. A moderately negative impact on Caco-2 lipid absorption was observed for PHM compared with RHM. Moreover, a lower mean lipid droplet size was observed in cells incubated with PHM, the physiological relevance of which remains to be elucidated.

CRediT authorship contribution statement

Marine Vincent: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Olivia Ménard:** Conceptualization, Methodology, Validation, Investigation, Writing - review & editing, Visualization. **Julie Etienne:** Methodology, Investigation. **Jordane Ossemond:** Methodology, Investigation, Visualization. **Annie Durand:** Investigation. **Rachel Buffin:** Investigation, Resources. **Emmanuelle Loizon:** Methodology.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126927.

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