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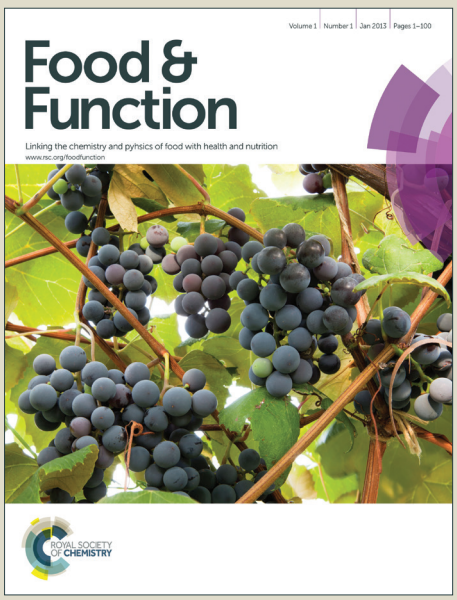

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ARTICLE

Impact of various emulsifiers on ALA bioavailability and chylomicron synthesis through changes in gastrointestinal lipolysis

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Formulate healthy food rich in omega-3 fatty acids requires prior the knowledge of the parameters influencing their bioavailability and their metabolic fate. In this context, we studied the effects of various emulsifiers widely used in food industry, on the gastrointestinal lipolysis of flaxseed oil emulsions in an *in vitro* model and on the intestinal absorption and lymphatic secretion of alpha-linolenic acid (ALA) in rats. *In vitro* data showed that the emulsification of flaxseed oil with soya lecithin improved the gastric lipolysis of the oil (+30%), while the presence of Tween 80 or of Sodium caseinate decreased it (-80% and -40%, respectively). The *in vivo* data demonstrated that the intestinal absorption and the lymphatic secretion of ALA was improved with soya lecithin (Cmax = 24 mg/mL) and reduced in presence of Sodium caseinate (Cmax = 7 mg/mL) compared to unemulsified flaxseed oil (Cmax = 16 mg/mL); Tween 80 had no effect. Besides the synthesized chylomicrons were notably larger and more numerous with soya lecithin whereas they were smaller in presence of Sodium caseinate ($p<0.05$). This study concluded that the intestinal bioavailability of ALA was increased by the emulsification of flaxseed oil with soya lecithin via an improved lipolysis favouring the intestinal absorption of ALA and the secretion of many large chylomicrons in lymph.

Introduction

Alpha-linolenic acid (ALA) is the dietary precursor for the long-chain omega-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). ALA is associated to lower cardio cardiovascular risk (CVR) through various biologic mechanisms, including platelet function, inflammation, endothelial cell function, arterial compliance, and arrhythmia[1–5] Epidemiological studies have highlighted an unsufficient intake of omega-3 PUFA in Western countries, particularly with regard to ALA[6]. In this context, authorities advise to increase the dietary consumption of ALA to reach 2 to 3 g per day for the primary and secondary prevention of CVR[7].

It has been reported that ALA follows essentially the β-oxidation pathway in the liver[8]. In contrast, little information exists regarding its intestinal bioavailability, *i.e.*, uptake and reassembly into lymph chylomicrons (CM). Only a few works have demonstrated that the bioavailability of ALA was notably improved by the formulation of linolenic oils. Especially, the emulsification process has been shown to enhance ALA bioavailability in human plasma[9,10] and in rat lymph[11,12] with phospholipids (PL) from lecithin, used as surfactants. Actually, the existence of lipid droplets preformed by the emulsification seems to modulate lipid absorption by enhancing gastrointestinal lipolysis during meal digestion tests in healthy volunteers[13,14]. Indeed the interface created to stabilize the lipid droplets could modify the digestion of the emulsion by modulating the access of lipolytic enzymes: gastric and pancreatic lipases[15,16]. This could explain why emulsified fat is absorbed and metabolized faster than the same unemulsified fat[13,17,18]. Besides, interface composition, *i.e.* the nature of emulsifiers, can affect the metabolism of lymph CM[19] and then plasma triacylglycerol (TAG) concentrations[20]. Indeed, the improvement of intestinal fatty acid absorption would result in a modification of lipid micellisation[21] and would promote the accretion of TAG in chylomicrons (CM) in rat lymph[22,23,24] and in Caco-2 cells[25]. The characteristics of chylomicrons, synthesized in the enterocyte, seemed to impact lipemia in plasma. Especially an excessive postprandial lipemia during digestion is associated with an increased risk of cardiovascular disease. Moreover, for a given amount of TAG, small CM would be more atherogenic than large ones due to the limiting rate of chylomicron TAG hydrolysis by the lipoprotein lipase at the vascular wall[25,26].

Therefore, when attempting to formulate essential fatty acid (FA) sources, besides the impact of FA absorption, the properties of chylomicrons must be also taken into account, particularly with respect to the increasing use of emulsifiers in the human diet[27]. To our knowledge, no data is available up to date regarding the physical properties of lymph CM during the

postprandial phase after absorption of differently structured emulsions.

In this context we hypothesized that some commonly emulsifiers used in food industry could modulate the gastrointestinal lipolysis step and subsequently modify (i) the recovery of ALA, (ii) the intestinal production of CM and (iii) their structural properties. To this aim, we explored, in rodent model, the effects of different types of surfactants (Sodium caseinate, Tween 80 and PL), used to emulsify flaxseed oil, on (i) its lipolysis rates, (ii) the kinetics of ALA absorption and reassembly into lymph CM and (iii) physical characteristics of lymph CM. We assumed that according to the efficiency of the lipolysis of flaxseed oil emulsion, ALA absorption would be modulated as well as the properties of lymph CM.

Experimental

Materials.

Flaxseed oil was supplied by ITERG (Pessac, France). Fatty acid composition was defined according to the method of Vigneron *et al.*[28] and fatty acid methyl esters (FAME) were analyzed by gas chromatography (GC) coupled to a flame ionisation detector (FID) as previously described[11]. A commercial soya lecithin (Lecimulthin) was kindly provided by Cargill (Baupte, France). The lecithin was mainly composed of phosphatidylcholine (13%, v/v), phosphatidylethanolamine (30%; w/w), phosphatidylinositol (25% w/w) and Lyso-PL (23%). Sodium caseinate and Tween 80 were purchased from Sigma (St Louis Mo, USA). Lipolytic enzymes were provided by EIPL-CNRS; recombinant dog gastric lipase (rDGL) (>98% protein purity) was produced by Meristem Therapeutics (Clermont-Ferrand, France). rDGL stock solutions at 1 and 0.1 mg rDGL/mL were prepared in 10 mM 2-(N-Morpholino) ethane sulfonic acid sodium salt (MES), 150 mM NaCl, (pH 6.0). Porcine pancreatic extracts (PPE or pancreatin) were used as a source of pancreatic lipase (5.8 mg lipase /100mg PPE; equivalent to 174 USP lipase units/mg). PPE also contained active proteases (≥25 USP units/mg) and amylase (≥25 USP units/mg). Bovine bile extract and lipid standards (trimyristolein, triolein, diolein, monolein oleic acid and 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine) were obtained from Sigma (St Louis Mo, USA); purety >98%. All the solvents were of analytical grade and purchased from SDS (Peypin, France) and from Sigma (St Louis Mo, USA).

Preparation and characterization of the O/W emulsions.

Three Oil-in-Water (O/W) emulsions from flaxseed oil were manually prepared at room temperature under a nitrogen flux to prevent lipid oxidation. The aqueous phase of Sodium caseinate and Tween 80 emulsions contained respectively 12% of Sodium caseinate and 5% Tween 80 in distilled water (v/v), so that the oil phase represented 70% of the emulsion (v/v). While for the lecithin emulsion, the soya lecithin was dispersed in oil phase (33%; w/v), so that the oil fraction represented 25% of the final emulsion (v/v). Lipid formulations were emulsified using an Ultraturax apparatus [Janke & KunKel, equipped with a generator axis (10 mm S25-N-10G - IKA)]. The coarse O/W emulsions obtained were then sheared in a Couette cell (concentric cylinders geometry, Ademtech SA, France), with a gap of 100 µm, 600 RPM. Mean particle diameter (as evaluated by the volume weighted average diameter d*4,3*and the surface weighted average diameter d*3,2*) and particle size distribution were determined by static light-scattering, using a Coulter LS 230 apparatus. The droplet size distributions were close for the three emulsions. The mean diameter (d*4,3*) of lipid droplets was found to be 5 µm for the soya lecithin and Tween 80 emulsions and 4 µm for the Sodium caseinate one (Table 1). Distribution of droplets is tightened with respect to Sodium caseinate and Tween 80, whereas a second minority population of lipid droplets was observed for the lecithin emulsion and of mean diameter 10 µm. The total fatty acid compositions of the three O/W emulsions (Table 1) were obtained following the procedure of Lepage and Roy[29] and defined by analyzing fatty acid methyl esters (FAME) by GC-FID, as previously described[11].

Table 1. Main fatty acid profile (mol%) and droplet size distribution of O/W flaxseed oil emulsions stabilized by Tween 80 (2%), or by a soya lecithin (8%) or by Sodium caseinate (4%).

	Flaxseed oil	Tween 80	Soya Lecithin	Sodium caseinate
	Fatty acid profile *(mol%)*			
SFA	10.1	10.1	9.9	10.1
16:0	5.1	5.1	5.4	5.1
18:0	4.0	4.0	4.0	4.0
MUFA	20.8	20.8	20.1	20.8
18:1 *n-9*	18.5	18.5	19.0	18.5
n-6 PUFA	16.1	16.1	19.7	16.1
18:2 *n-6*	15.5	15.5	19.6	15.5
n-3 PUFA	46.0	46.0	47.2	46.0
18:3 *n-3*	46.0	46.0	47.2	46.0
	Droplet size distribution *(µm)*			
d*4,3*	-	4.8	5.5	3.7
d*3,2*	-	3.4	3.1	3.0
Specific surface area	-	17,543	19,084	20,048

Fatty acid composition and droplet size distribution represent the mean of two measurements. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. **P*<0.05. ANOVA followed by Fisher test.

In vitro gastric and intestinal lipolysis of flaxseed oil in bulk phase and in emulsions.

The lipolysis of flaxseed oil and of the three emulsions was tested using a two-step *in vitro* digestion model mimicking the physiological conditions found in the gastrointestinal (GI) tract of healthy human adults (lipase concentrations, pH values, dilution factors) at half-gastric emptying of a test meal[13]. For initiating the gastric step of lipolysis, 1 g of each emulsion was dispersed in 15 mL of an assay solution containing 150 mM NaCl, 1.4 mM $CaCl_2$, 10 mM MES. This mixture was mechanically stirred in a temperature-controlled reaction vessel at 37°C and the pH was adjusted to 5.5. At t=0 min, 3 mL of a freshly prepared rDGL solution at 0.1 mg/mL were added to the reaction vessel to obtain a final concentration of 17 µg/mL of rDGL and the pH was kept constant at 5.5 during 30 min, via an automated titration of free fatty acids (FFA) with 0.1 M NaOH using a pH-stat device. The duodenal step of lipolysis was launched at t=30 min by adding 11 mL of PPE/bile salts solution to the mixture and the pH was shifted to 6.25 and then kept constant for 60 min. After adding the pancreatic enzyme solution to the reaction vessel, the final pancreatic lipase

concentration was 250 µg/mL, the bile salt concentration was 4 mM and the gastric phase was diluted 1.7-fold. Samples (1 mL) were collected at t=0 min, 15 min, 29 min, 35 min, 40 min, 45 min, 60 min, and 90 min for lipid extraction and analysis.

Extraction and quantitative analysis of lipolytic products.

Lipid extraction was performed immediately after sampling by mixing vigorously each 1 mL with 200 µL 0.1 N HCl and 5 mL chloroform-methanol (2:1 vol/vol) in a 15 mL glass tube with a screw-cap. After phase separation, the lower organic phase was collected using a Pasteur pipette, transferred to a 15-mL test tube, and dried over anhydrous magnesium sulfate ($MgSO_4$). After total precipitation of $MgSO_4$, the clear organic phase was filtered and transfered into a 2 mL vial with a screw-cap and the vial was kept at –20°C until the analyses were performed. The quantitative analysis of residual TAG and lipolysis products (FFA, diglycerides (DAG) and monoglycerides (MAG) was performed by thin-layer chromatography coupled to flame ionization detection (TLC-FID technique) using Iatroscan MK6 equipment (Iatron Laboratories)[30]. Each lipid extract (5 µL) was spotted onto a quartz rod coated with silica (0.9 mm diameter Chromarod™ SIII, Iatron Laboratories) and sample migration was performed with heptane/ether/formic acid (55:45:1 v/v/v). Chromarods were dried at 150°C for 15 min (Rod dryer TK8, Iatron Laboratories) and transfered to the Iatroscan MK6 to be scanned by the FID for detection and quantification of the compounds separated on silica. Known amounts (0.1 to 10 µg) of reference standard compounds (triolein, diolein, monolein, oleic acid) were used to calibrate the mass detection by FID. For each class of compound analyzed, a calibration curve (peak area *vs* mass) was established and used to quantify the lipid masses in the various samples analyzed. The mass detection data was converted into moles. The hydrolysis level (% FFA versus total acyl chains) of flaxseed oil and various emulsions was calculated at each time point and the variations in all molecular species were plotted as a function of time.

Animals and surgical procedures.

Male Wistar rats (8 weeks-old, body weight 300-350 g) were obtained from Elevage JANVIER (Saint-Berthevin, France) and were randomly assigned to one of the dietary groups. The study was conducted in accordance with European Community Council Directives (861609/EEC). All experiments conformed to the Guidelines for the Handling and Training of Laboratory Animals. Rats were housed for at least 3 days before the experiment in a controlled environment, with constant temperature and humidity with free water and food access. Rats were fed a fat-free diet (SAFE, Epinay, France) with free access to water 24 h before the surgery. Each rat was placed under slight isoflurane anesthesia (1 L oxygen flow containing 0.25% of isoflurane solution) and a polyethylene catheter (i.d. 0.86 mm, o.d. 1.27 mm, Biotrol, Paris, France) was inserted into the main mesenteric lymph duct[31]. After surgery, rats were placed in individual restraining cages, in a warm environment with tap water freely available.
Lymph collection experiments were performed after intragastric gavage of flaxseed oil, either in bulk phase or emulsified with one of the three surfactants indicated. In each experiment, 300 mg of lipids were administered equivalent to 120 mg of ALA. Lymph was collected at 1 h intervals for the next 6 hours post feeding. During the collection period, the lymph flow averaged 0.4 ± 0.1 mL/h. At least 8 rats were cannulated per group and, killed by an overdose of sodium pentobarbital after sampling.

Total fatty acid and ALA recoveries in lymph chylomicrons.

The total fatty acid profiles in lymph were determined according to the method of Lepage and Roy[29]. Trimyristolein was added as an internal standard for fatty acid quantification. FAME were analyzed by GC on a BPX 70 capillary column (60 m long, 0.25 µm film, 0.25 mm i.d., SGE, hydrogen as carrier gas, split ratio of 1:80). The GC system consisted of a gas chromatograph Focus GS (Thermofinnigan, Courtaboeuf, France) equipped with a flame ionization detector maintained at 250°C. The injector temperature was 250°C. The column temperature was increased from 150°C to 200°C (1.3°C/min), maintained at 200°C for 20 min, increased from 200°C to 235°C (10°C/min), and held at 235°C for 20 min. Data handling was performed by Chromquest software (Thermofinnigan, Courtaboeuf, France). Fatty acids from Sigma France (St Louis Mo, USA) of known composition were used as standards for column calibration. The variation in peak area between injections was less than 2%.

Size and number of lymphatic chylomicrons.

The size of lymph chylomicrons was measured in each diet group throughout the kinetics of lipid absorption using 20 µL of lymph collection. The samples were first diluted in a filtered (0.2 µm) NaCl solution in water (9 $g.L^{-1}$; w/v). The initial diameter of lymphatic chylomicrons was analyzed by static light-scattering using Nanosizer N4+ (Beckman Coulter, California, USA). The quantification of chylomicron particles was obtained by the measurement of lymph Apo-B48 by using the rat Apo-B48 ELISA-kit according to the manufacturer's instructions (Cusabio, Aachen, Germany).

Real-time quantitative RT-PCR analysis.

The first 30 cm of the small intestine were split in two lengthwise, rinsed with cold NaCl solution (9 $g.L^{-1}$; w/v, 4°C). The enterocyte cells were scraped with a glass slide and immediately frozen in liquid nitrogen and stored at −80°C. Samples were pulverized in liquid nitrogen and 100 mg were used for RNA extraction. Total RNA extraction was performed using Tri Reagent™ (Applied Biosystems) following manufacturer's procedure. RNA concentration was measured with Nanodrop ND1000 (Labtech, Palaiseau, France). First-strand cDNA were synthesized from 1 µg of total RNA with PrimeScript RT reagent Kit (Takara) following manufacturer's procedure. Real-time PCR assays were performed as previously described[32], using a Rotor-Gene 6000 (Qiagen, Courtaboeuf, France). In a final volume of 20 µL containing 5 µL of a 60-fold dilution of the reverse transcription (RT) reaction medium, 10 µl of reaction buffer from the Absolute QPCR SYBRGreen ROXMix (Abgene, Courtaboeuf, France) and 0.375 µM of the specific forward and reverse primers. For quantification, a standard curve was systematically generated with six different amounts of cDNA. Each assay was performed in duplicate, and validation of the realtime PCR runs was assessed by evaluation of the melting temperature of the products and by the slope and error obtained with the standard curve. Hypoxanthine phosphoribosyltransferase (HPRT) mRNA level was determined in each sample and was used as internal standard for normalization of target mRNA expression. The PCR primers are available upon request.

Statistical analysis.

Data were expressed as mean values with their standard errors. ALA bioavailability was estimated by the area under the curve (AUC) and calculated according to the trapezoidal method. Data were analysed by one-way ANOVA followed by post hoc Fisher's test (i) to evaluate the influence of the nature of different emulsifiers on the gastric and intestinal lipolysis levels of flaxseed oil, (ii) to compare the total fatty acid and ALA concentrations in lymph (iii) to appreciate the physical characteristics of lymph chylomicron and (iv) to compare the expression of genes encoding for the proteins involved in the absorption and fate of fatty acids within the enterocyte. Only the above tests with significance at the $P<0.05$ level were judged to be significant.

Results

The type of emulsifier affects differently the *in vitro* gastrointestinal digestion of flaxseed oil.

Gastric and duodenal lipolysis of flaxseed oil (bulk form or emulsified) were tested *in vitro* using a two-step static digestion model involving a first incubation with gastric lipase (rDGL) followed by the addition of pancreatic extracts (Fig. 1). The lipolysis levels were estimated from the quantitative analysis of FFA, MAG, DAG and residual TAG. Gastric lipolysis of flaxseed oil by rDGL at 30 min was increased 1.3 fold by emulsification with soya lecithin, while it was decreased 1.7 fold and 5.8 fold when Sodium caseinate and Tween 80 were used as emulsifiers, respectively (Table 2). Only differences between soya lecithin-emulsion and casein- or Tween-stabilized emulsions were found to be significant ($P<0.05$). The same ranking in lipolysis rates was conserved during the duodenal phase of lipolysis with the soya lecithin emulsion, presenting the highest lipolysis level at 90 min (50.3%> flaxseed oil, 39.7%> Sodium caseinate-flaxseed, 32.6%> Tween 80-flaxseed, 28.9%). In this case, the difference between soya lecithin- emulsion and unemulsified flaxseed oil was also found to be significant ($P<0.05$).
Interestingly, the deviation between the lipolysis rates of Tween 80 emulsion and flaxseed oil at 30 min (-10%) was conserved at 90 min, thus indicating that the presence of Tween 80 drastically impaired the gastric step of lipolysis but had no significant effect on the rate of duodenal lipolysis by pancreatic lipase (Fig. 1).

The ALA accretion in lymph is modified by the nature of the emulsifiers.

The kinetics of ALA recovery in rat lymph was followed during 6 h after gavage (Fig. 2). For most groups, the ALA absorption followed typical sigmoidal kinetics and the recovery of ALA in lymph increased from 1 h onwards, reflecting the beginning of the absorption step. The kinetics of ALA recovery was similar between flaxseed oil and Tween 80 groups. However when flaxseed oil was emulsified with soya lecithin, the secretion of ALA in lymph was significantly enhanced at 5 h (24±3 mg/mL), when the absorption had plateaued, compared to the other groups (flaxseed oil, Tween 80 and Sodium caseinate; 16±7, 13±6 and 6±2 mg/mL, respectively). Conversely, the absorption and secretion of ALA in lymph was greatly limited using Sodium caseinate. Indeed, the C_{max} value in Sodium caseinate group was 60% lower than the C_{max} value in flaxseed oil group (7±4 mg/mL, $P<0.05$).
The intestinal absorption of ALA over the entire period is evaluated from the AUC obtained from kinetics of ALA absorption (Insert Fig. 2). The AUC was similar between flaxseed oil and Tween 80 groups (on average, 52 mg/mL.h). However, the AUC of ALA was nearly 40% lower in Sodium caseinate group compared to flaxseed oil and Tween 80 groups, whereas it was dramatically increased (+120%) in lecithin group ($P<0.05$).

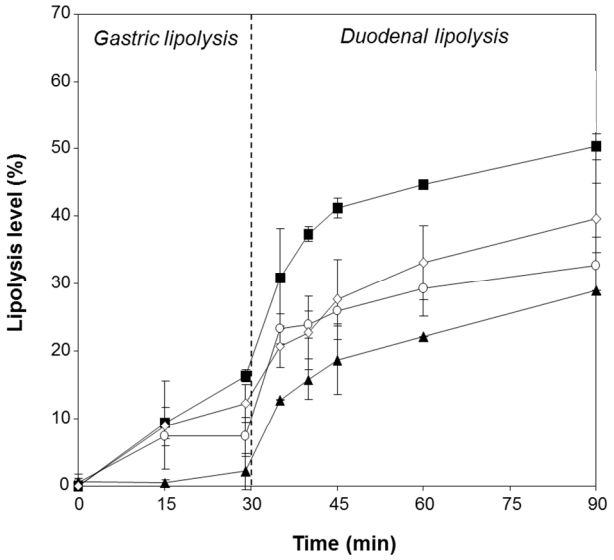

Fig. 1 Lipolysis levels of flaxseed oil using a two-step *in vitro* digestion model, in bulk phase (◇) or emulsified with Sodium caseinate (○), soya lecithin (■) or with Tween 80 (▲). The lipolysis levels were estimated from the TLC-FID (Iatroscan) analysis of lipolysis products. Lipids were separated by a single migration with heptane/ether/formic acid (55:45:1 v/v/v) before FID detection. Lipolysis levels are expressed as % FFA versus total acyl chains present in residual TAG, DAG, MAG and FFA. Values are means ± SD (n=3).

Table 2 Lipolysis levels of flaxseed oil in bulk phase or emulsified under *in vitro* conditions mimicking the gastric and duodenal phases of digestion.

Oil / Emulsion	Gastric lipolysis level (%) at 29min	Duodenal lipolysis level (%) at 90min
Flaxseed oil (unemulsified)	12.2 ± 2.7	39.7 ± 5.1
Sodium caseinate-emulsion	7.3 ± 2.9	32.6 ± 4.3
Tween 80-emulsion	2.1 ± 2.7	28.9 ± 0
Soya lecithin-emulsion	16.3 ± 0.8	50.3 ± 2.0

Lipolysis levels are expressed as % FFA *vs* total acyl chains present in residual TAG, DAG, MAG and FFA. Values are means ± SD (n=3). For a given lipolysis step (gastric or duodenal), lipolysis levels presented without a common letter were significantly different ($P <$ 0.05; ANOVA followed by Fisher's test).

The total fatty acids uptake is modulated *in vivo*, by the nature of emulsifiers.

The export kinetics of total fatty acids into lymph compartment was followed during 6 hours after intragastric gavage of lipid

formulations (Table 3). The secretion of fatty acids in lymph was significant from 4 h and maximum concentration in lymph (C_{max}) was reached at 5 h, regardless of the group (unemulsified or emulsified flaxseed oil). The C_{max} was similar for both flaxseed oil and Tween 80 groups and the highest for lecithin group (P<0.05). Only Sodium caseinate group presented a linear kinetics, which characterized a poor lipid absorption, confirmed by the lowest C_{max} (P<0.05).

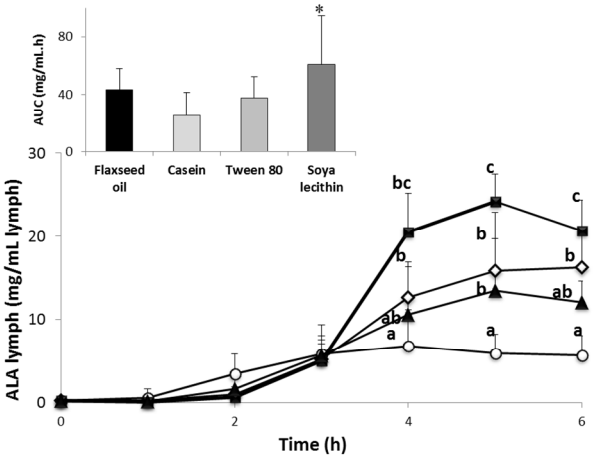

Fig. 2 Time course concentration of ALA in lymph of rats fed with flaxseed oil either in bulk phase (◇) or emulsified with Sodium caseinate (○), soya lecithin (■) or Tween 80 (▲). Values represent mean of at least 8 rats for each lipid system, at each time point. From 4 h, all points differ significantly from baseline. For a given timepoint, concentrations presented without a common letter were significantly different (P < 0.05; ANOVA followed by Fisher's test). Insert represents the corresponding area under the curve (AUC) *P<0.05 *vs* other groups (ANOVA followed by Fisher test)

Physical characteristics of lymph chylomicron depend on the type of emulsifiers.

Physical characteristics of lymph CM, *i.e.*, diameter and number of particles were presented at 2 h and for the period enclosing the C_{max} value (4 h -6 h) (Table 3). Overall, the maximal diameter (d_{max}) of CM was observed at 5 h for most groups, which was consistent with the time for which the C_{max} value was obtained.

The d_{max} observed in flaxseed oil group was similar to those observed for lecithin and Tween 80 groups. Conversely, the d_{max} value in the Sodium caseinate group was lower than those observed in the other groups (P<0.05). It is noteworthy that the CM diameter observed at T_0 (C_0=176.1 ± 31.3 nm on average, data not shown), indicated that the diameter of the CM has been doubled 4 hours after lipid ingestion.

Besides, the number of CM particles was obtained by the concentration of ApoB48 in lymph. The number of lymph CM in lecithin group was twice higher 6 hours post feeding than the number of CM in flaxseed oil group and Tween 80 group, but similar to the CM number in casein group.

Emulsifiers impact gene expressions involved in the intestinal fatty acid absorption and lymph chylomicron assembly and secretion.

The expression of the intestinal fatty acid binding protein (FABP2) gene involved in the absorption of lipids within the enterocyte and those of the microsomal triglyceride transfer protein (MTTP), apolipoprotein B48 (ApoB48) and Sar1b involved in the compaction, formation and exocytosis of CM respectively, were estimated at 6 h post-feeding in the small intestine. Flaxseed oil and lecithin groups presented increased expression of FABP2 (+180%) *vs* ungavaged control rats, while Sodium caseinate group presented lower FABP2 expression (-50%) than both flaxseed oil and lecithin groups (Fig. 3).

Lecithin group was the only group in which ApoB48 gene was overexpressed (+100%) compared to ungavaged control group. Regarding chylomicron assembly and transport, lecithin group presented higher expression of both MTTP (+150%) and Sar1b (+130%) *vs* control, while Sodium caseinate group presented lower expression of the latter genes *vs* lecithin group (-51 and -35%, respectively). Altogether, emulsification using lecithin presented the most significant enhancing effect towards the expression of various genes involved in the enterocyte lipid absorption cascade.

Discussion

This study evaluated *in vitro* and *in vivo*, the impact of several food emulsifiers on (i) the gastrointestinal lipolysis of flaxseed oil, (ii) the intestinal bioavailability of ALA based on its release in lymph and, (iii) the postprandial secretion of CM and their physical properties. The emulsifiers, differing in their biochemical nature, were either protein (Sodium caseinate), polar lipid (soya lecithin) or a small molecular weight synthetic, nonionic surfactant (Tween 80).

In this study, we have demonstrated that the nature of the food emulsifiers differently affected the gastrointestinal lipolysis rates by gastric and pancreatic lipases, the intestinal uptake of ALA by the enterocyte and its export in lymph compartment. Moreover, we have shown that the characteristics of lymph CM were modulated by the trans-enterocyte flux of lipids according to a two step-process; one qualitative concerning the diameter of lymph CM, and the other quantitative about the CM particle number.

The intestinal uptake of ALA was characterized both kinetically and by its overall absorption (AUC) on the 6 hour-kinetics. Moreover, absorption mechanism was studied *via* the expression, in the duodenum, of gene encoding for FABP2 involved in fatty acid transport in the enterocyte. In the *in vivo* study, ALA absorption in the enterocyte was significantly enhanced in lecithin group (+50%) compared to flaxseed oil group, which was consistent with the high expression of FABP2 observed. On the contrary, Sodium caseinate appeared to reduce the absorption of ALA (-37%). Moreover, Sodium caseinate induced a flat kinetic of ALA absorption, unlike the sigmoidal ones observed in the other groups. This flat curve suggested that a lower trans-flux of ALA occurred in the enterocyte, which appeared consistent with the decrease of FABP2 gene expression observed in Sodium caseinate group (-50%).

Up to date, only few studies dealt with the impact of different emulsifiers on FA absorption. Some enhancing properties of emulsifying the oil with PL were reported regarding the intestinal absorption of fatty acids in rats, compared with unemulsified oil of vegetable or animal origins[21–23,33,34].

These data were mainly discussed according to the amount of oil/water interface generated by emulsification. Indeed, the interface plays an important role on GI lipolysis because the former is necessary for the access and activity of lipolytic enzymes on their substrate[14,15].

Table 3 Total fatty acid recovery and chylomicron particle analysis in rat lymph at 4, 5 and 6 h after gastric tube-feeding of flaxseed oil formulations

Time (h)	Lipid formulation	Total FA in lymph (mg/mL)	Chylomicron diameter (nm)	ApoB48 (µg/mL)
2	Flaxseed oil (unemulsified)	$7.2^{ab} \pm 1.4$	$206.0^{a} \pm 51.4$	$675.4^{a} \pm 60.0$
	Lecithin-emulsion	$4.6^{b} \pm 2.5$	$208.1^{a} \pm 30.7$	$648.5^{a} \pm 58.0$
	Tween 80-emulsion	$8.8^{a} \pm 3.3$	$211.8^{a} \pm 51.7$	$310.6^{b} \pm 71.9$
	Sodium caseinate-emulsion	$8.7^{ab} \pm 5.2$	$216.6^{a} \pm 52.7$	$639.3^{a} \pm 11.6$
4	Flaxseed oil (unemulsified)	$30.5^{a} \pm 11.7$	$294.9^{a} \pm 20.2$	$494.7^{a} \pm 61.7$
	Lecithin-emulsion	$21.5^{a} \pm 15.3$	$298.1^{a} \pm 33.9$	$605.0^{a} \pm 72.3$
	Tween 80-emulsion	$25.0^{a} \pm 10.9$	$293.6^{a} \pm 45.9$	$478.3^{a} \pm 71.7$
	Sodium caseinate-emulsion	$17.2^{a} \pm 8.4$	$254.7^{a} \pm 33.8$	$595.5^{a} \pm 46.8$
5	Flaxseed oil (unemulsified)	$36.9^{a} \pm 16.5$	$297.7^{a} \pm 16.8$	$403.1^{a} \pm 32.9$
	Lecithin-emulsion	$46.4^{b} \pm 13.1$	$305.6^{a} \pm 18.7$	$809.1^{b} \pm 93.1$
	Tween 80-emulsion	$29.8^{a} \pm 11.8$	$302.2^{a} \pm 40.7$	$344.2^{a} \pm 52.5$
	Sodium caseinate-emulsion	$17.4^{a} \pm 2.8$	$240.5^{b} \pm 41.3$	$537.0^{ab} \pm 65.5$
6	Flaxseed oil (unemulsified)	$34.2^{ab} \pm 9.1$	$312.6^{a} \pm 17.2$	$459.5^{a} \pm 48.4$
	Lecithin-emulsion	$46.1^{a} \pm 16.8$	$299.0^{a} \pm 28.3$	$887.7^{b} \pm 63.2$
	Tween 80-emulsion	$28.6^{bc} \pm 7.8$	$300.0^{a} \pm 30.9$	$400.5^{a} \pm 42.8$
	Sodium caseinate-emulsion	$17.9^{c} \pm 4.1$	$224.9^{b} \pm 48.5$	$719.1^{b} \pm 39.8$

Data represent an average of two different determinations, n=8 per group. Means within a row lacking a common superscript differ *P<0.05. ANOVA followed by Fisher's test. Lipid ingestion represents 0.3 g by rat of flaxseed oil in bulk phase or emulsified with lecithin (8%), or Tween 80 (12%) or Sodium caseinate (4%).

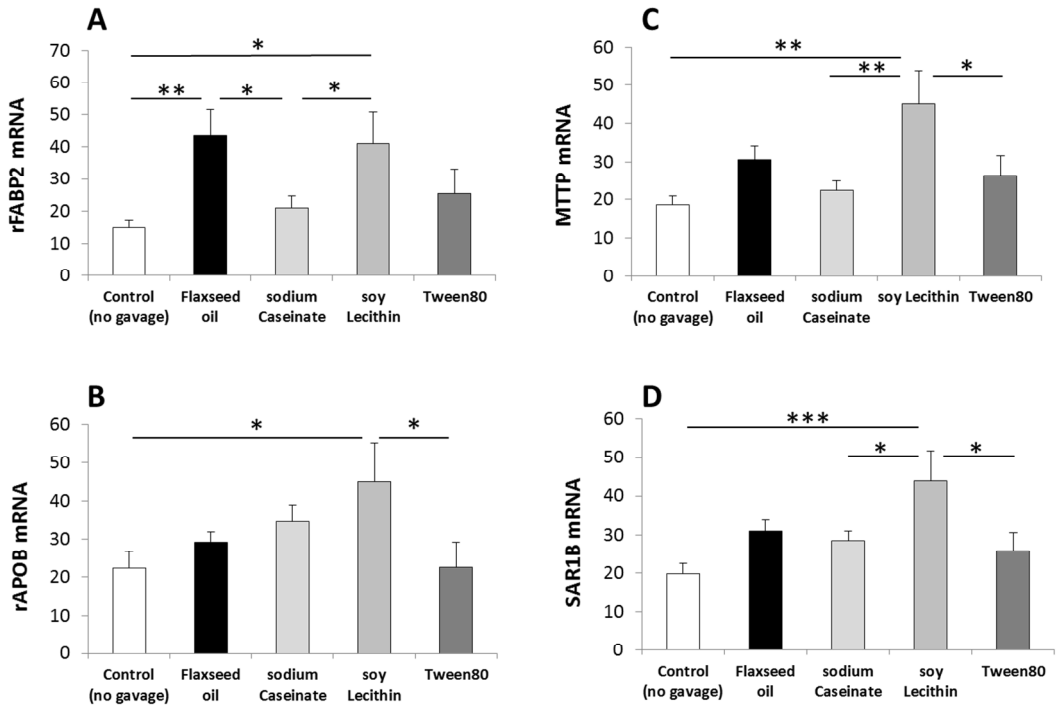

Fig. 3 Relative expression of mRNA of genes encoding for proteins involved in lipid absorption, relative to the endogenous HPRT gene, in the duodenum of rats collected 6 h after feeding with flaxseed oil, or emulsions stabilized with Sodium caseinate, soya lecithin or Tween 80, or after no gavage: (A) fatty acid transporter FABP2, (B) apolipoprotein ApoB48, (C) microsomal triglyceride transfer protein MTTP, (D) SAR1 homolog B Sar1b. n=7 per group. * P<0.05 (ANOVA followed by Fisher's test).

Because emulsified oil presents a dramatically increased surface area for lipolytic action compared with the same amount of bulk oil, the enhanced intestinal absorption of emulsified lipids can be explained by this stimulated GI lipolysis[17]. Moreover, the surfactants used as emulsifiers can also be involved in the solubilization of lipolysis products, like bile salts, and thus could modulate lipolysis rates by removing the lipolysis products from the oil-water interface[35–37]. Regarding the impact of these luminal mechanisms, micellar solubilization is also a critical step for the uptake of fatty acid by the enterocytes[38]. In the present study, the nature of emulsifiers mainly modified gastric lipolysis of flaxseed oil while duodenal lipolysis was poorly affected. The differences in overall lipolysis rates were thus mainly due to an increase (lecithin) or a decrease (Sodium caseinate, Tween 80) in gastric lipolysis. Assuming that lipolysis rates of flaxseed oil would be similar *in vivo*, the subsequent absorption rate of ALA was expected to be faster with lecithin than with Sodium caseinate and Tween 80. This was in fact consistent with the relative absorption of ALA observed *in vivo,* suggesting a role of enhanced lipolysis by lecithin-emulsification on the increased fatty acid absorption. These results were consistent with our previous data showing a faster lipolysis of lecithin-stabilized emulsions of rapeseed oil or milkfat compared with that of Sodium caseinate-stabilized emulsions[39], confirming that lecithin-emulsification provided an enhancing effect on lipolysis regardless of oil or fat composition.

Keogh et al.[20] previously observed in humans fed with Sodium caseinate-monoglyceride-emulsion, a lower postprandial rise in plasma TAG concentrations compared with lecithin or sodium sterol lactylate emulsions. In this latter work, the hypothesis of a delayed lipolysis of the Sodium caseinate-monoglyceride emulsion leading to a delayed intestinal absorption has been suggested. Indeed, our results showed a less efficient lipolysis of Sodium caseinate-stabilized emulsion *vs* lecithin-stabilized emulsion. However our results did not show a delayed absorption with Sodium caseinate but rather a lower absorption rate of lipids with Sodium caseinate compared to lecithin surfactant. Thus the limited ALA uptake observed in Sodium caseinate group resulted from a reduced lipolysis rate of the emulsion and could also be explained by a limiting micellar solubilization of fatty acids possible due to the protein-binding effect[40].

Moreover, it is noteworthy that the overall absorption of ALA was similar in flaxseed oil and Tween groups whereas the lipolysis rate of Tween 80-emulsion was much less effective than that of flaxseed oil (-83%). Tween 80 is known to inhibit gastric lipase activity[41,42] but could also favour the solubilization of lipolysis products in mixed micelles. In that case, the emulsifier might first delay lipolysis by impairing gastric lipase activity but then promote intestinal absorption of ALA by improving its solubilization in micelles. There is also an apparent contradiction between the slower lipolysis of Tween-emulsion *versus* casein-emulsion while ALA absorption is faster and more efficient with Tween than with casein. This discrepancy might result from the *in vitro* digestion model used in this study. This model focusing on lipolysis by gastric and pancreatic lipases does not contain pepsin in the gastric phase. The impact of casein proteolysis on flaxseed oil lipolysis cannot be observed under these conditions. *In vivo* data on ALA intestinal absorption suggest however that the digestion of casein- emulsion might be less efficient than that of Tween-emulsion and that proteolysis might have a negative impact on gastric lipolysis.

Besides the parameters that influence the intestinal uptake of ALA, it is noticable that ALA transport in enterocyte and export in lymph may also be affected by the mechanisms occurring in the enterocyte, namely: resynthesis of TAG and packaging in pre-CM. During the postprandial phase, fatty acids and 2-MAG derived from the intestinal hydrolysis of TAG are synthesized in new TAG by the main pathway of MAG. ApoB48 and MTTP are known to be central to CM assembly and Sar1b for their secretion as lipoprotein[43–46]. After a dietary fat load, the increased TAG flux can be accommodated by an increased CM size and/or number[25].Because each CM contains a single ApoB 48 molecule[47–49], an increase of CM number can be correlated with an increase of ApoB48 concentration.

In our study, the increase of chylomicron diameter in the 4 early hours likely corresponded to an enrichment of chylomicron core with TAG (+ 50%) in flaxseed oil group. From 4 h onwards, when lipid flux has plateaued, a maximal steady state size of CM was reached, of ~300 nm on average. With the lecithin emulsion, when a large flux of digested lipids occurred, as revealed by enhanced lipolysis *in vitro*, the number of CM secreted in lymph exceeded that of the other groups. These results were consistent with the higher accumulation of fatty acids observed in rat lymph and the higher expression level of all genes involved in the formation and secretion of CM in lecithin group: MTTP, ApoB and Sar1b. The higher CM number through the increase of ApoB expression and secretion in the lecithin emulsion group could be explained by the effect of lyso-phosphatidylcholine on increasing ApoB expression and TAG secretion as observed *in vitro* with Caco-2 cells[50]. When Sodium caseinate was used as emulsifier, a lower trans-flux of lipids was observed followed by an increased synthesis of smaller chylomicrons. These results were consistent with the low concentration of fatty acids in lymph and the unmodified expressions of FABP2 and MTTP observed in rats provided with Sodium caseinate emulsion. Altogether, these data suggested that the size and number of CM can be modulated by (i) the trans-enterocyte flux rate of TAG generated during lipolysis step and (ii) the direct effect on the enterocyte of the lipolysis products. So, the synthesis of CM during the postprandial phase seemed to get started by a growing phase where particle diameter enhanced up to a maximal value, in excess of which, the number of particles was then increased. This latter step should occur for a greater compaction of the fatty acid in CM and to overcome the large flow of incoming lipids. Conversely, when few lipids were uptaken by the enterocyte (Sodium caseinate group), the synthesis of many small lipoproteins occurred.

The potential impact of such differential intestinal absorption of ALA and associated CM structure should not be underrated. Indeed, recent results showed that a rapid postprandial kinetics of chylomicronemia, induced by fat emulsification, resulted in an enhanced beta-oxidation of the ingested fatty acids compared with unemulsified fat[18]. Therefore, the impact of flaxseed oil emulsion structure on the final metabolization of ALA remained to be elucidated. Moreover, observed variations in CM size could be of functional importance in the metabolic handling of lipoproteins derived from flaxseed oil. Indeed, previous reports showed that for a similar amount of TAG, large CM were cleared faster from the bloodstream than small ones[25,51,52], and could have health effect especially regarding to

cardiovascular risk. Our results therefore suggested that the metabolic clearance of CM from flaxseed oil could be modulated by the emulsion formulation, which would deserve further investigation.

Conclusions

In summary, we demonstrated that the nature of surfactants and emulsifying agents commonly used in human diet differently modified the gastric and duodenal lipolysis of lipid emulsions. In the case of linolenic oils, this alteration conditioned ALA absorption and modulated the enterocyte production of lymph CM rich in ALA. We showed that lymphatic bioavailability of ALA, from flaxseed oil, could be improved by soya lecithin or decreased with Sodium caseinate, particularly because of their antagonistic effects on the gastrointestinal gastro intestinal lipolysis. Similarly the synthesis of chylomicrons was redesigned in terms of number and size according to the flux of fatty acids generated during lipolysis. These novel findings could help for a better understanding of (i) the intestinal metabolism of the precursor of the omega-3 series and (ii) the lipoproteins in lymph in order to increase the bioavailability of n-3 fatty acids. Thus, the use of optimal formulations for the emulsification of linolenic oils could represent a nutritional benefit in the case of improving the status of omega-3, including ALA, and its benefits on CVR.

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Notes and references

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