SUPPLEMENTARY MATERIALS AND METHODS

CLINICAL STUDIES

The dietary intervention trial, namely the VALOBAB-C study, was a multicentre double-blind randomized trial with a parallel group design. The acute milk PL consumption study, namely the VALOBAB-D study, was a multicentre double-blind randomized trial with a crossover design. All participants provided informed, written consent prior to the clinical trials, which were both approved by the Scientific Ethics Committee of Lyon Sud-Est-IV (ID-RCBs: 2013-A01125-40 and 2013-A01467-38) and ANSM (French Agency for the Safety of Health Products). Trials were conducted at the Human Nutrition Research Centre Rhône-Alpes (CRNH-RA; Lyon, France) and the Human Nutrition Research Centre Auvergne (CRNH-A; Clermont-Ferrand, France). All studies were carried out in accordance with the Second Declaration of Helsinki and the French Huriet-Serusclat law. Both clinical trials were reported and registered on http://www.clinicaltrials.gov (VALOBAB-C: NCT02099032, VALOBAB-D: NCT02146339).

Study design

VALOBAB-C

The study was over a 5-week period starting with a run-in period of one week during which subjects had to consume daily 100 g of the Control cream cheese (non-enriched with MPL) (figure 1A, supplementary figure S1A). After a 1-week run-in period, the volunteers were randomly assigned to 3 different groups for a 4-week intervention period: a group consuming cream cheese non enriched in PL (Control group), a group consuming cream cheese enriched with 3 g of PL (3g-PL group) or a group consuming cream cheese enriched with 5 g of PL (5g-PL group). After this first week, fasting participants came early in the morning to the nutrition research centre after a 10h-fast for the first visit of metabolic explorations (V1, one

day long). Then, participants got a supply of 28 cream cheese portions (100 g each) to be kept refrigerated at home for the 4-week intervention period. At the end of the intervention period, participants came back to the nutrition research centre for the second visit of metabolic explorations (V2, one day long). All along the 5-week period, subjects were asked to continue their usual diet and physical activity, but with some exceptions. During the week before V1 (run-in period with Control cheese) and all along the 4-week intervention period, participants were told to (i) include the cream cheeses to their daily diet and avoid consumption of other cheeses, (ii) avoid listed foods very rich in fibres and foods that may influence microbiota (fermented products, light foods). For 24 h prior to V1 and V2, subjects were also asked to refrain from consuming alcohol and to avoid strenuous exercise. In addition, participants were asked to eat a standardized dinner developed by a dietician the evening prior to V1 and V2.

VALOBAB-D

Selected volunteers came to the nutrition research centre for three distinct days of metabolic testing separated by a washout period of 4 to 6 weeks (supplementary figure S2A). During each metabolic day, called visit 1 (V1), visit 2 (V2) and visit 3 (V3), a different breakfast was served including the test cream cheese containing either no PL (Control), 3g of PL or 5g of PL. During the protocol, all subjects were asked to continue their usual diet and activity except for the week before each test day. The subjects were told to avoid foods naturally rich in ^{13}C during the week before each exploration visit and were given a list of such foods. The day before and the day of each testing, participants were asked not to use anti-diarrheal medicines. For 24h before testing, the subjects were also asked to refrain from consuming alcohol and to avoid strenuous exercise. In addition, the subjects were provided with a standardized dinner on the evening before testing.

Study participants

VALOBAB-C

The phone pre-screening of candidates allowed selecting 141 subjects. Among them, 83 were excluded mainly because they did not meet the required biological criteria (notably the level of HDL-cholesterol) or have medical incompatibility (e.g. treatment interfering with lipid metabolism). Finally, 58 eligible volunteers were included in the study, 19 in the Control group, 19 in the 3g-PL group and 20 in the 5g-PL group. We could not extend the inclusion period with the aim to obtain the total sample size initially planned (see "Statistical analyses" section below) due to (i) the limited availability of the cream cheeses, that were produced on purpose every 5 months by a dedicated platform (see below Cream cheese section) and (ii) the expiry date of the ingredient used for PL-enriched cream cheeses. The ingredient with the unique concentration of 34 g milk PL per 100 g dry matter (see below Cream cheese section) was produced at the beginning of the study and could not be produced again at the end of the project. The 58 individuals followed the entire trial and their data were analysed (supplementary figure S1B).

All subjects had to meet specific inclusion criteria: age ≤ 75 y, BMI ranging from 25 to 35 kg/m², waist circumference greater than 80 cm, fasting total-cholesterol concentration < 11 mmol/L, fasting HDL-cholesterol concentration < 1.6 mmol/L, fasting triacylglycerol concentration < 3 mmol/L and fasting glycemia < 7 mmol/L. Non-inclusion criteria were CRP concentration > 20 mg/L, BP > 140/90 mmHg, ASAT and ALAT concentrations > 1.5 times normal values, dairy products intolerance, treatment interfering with lipid metabolism, hormonal treatment, evolving pathology, regular heavy exercise (> 3h/week), particular diet (e.g. vegetarian, vegan) and tobacco (> 5 cigarettes/day). Volunteers of the VALOBAB-C study were recruited through announcements on local intranet and media and through small posters in different work places in Lyon and Clermont-Ferrand (France). All participants

received written and oral information and their medical history was reviewed. In addition, they underwent a physical examination and fasting clinical analyses were performed before enrolment.

VALOBAB-D

The pre-screening of candidates by digestive surgeons and gastroenterologists allowed us to contact 40 individuals. Unfortunately, 36 of them were excluded mainly because they did not meet all inclusion criteria (taking treatment interfering with lipid metabolism or having another evolving pathology). Ultimately, only 4 volunteers could be recruited due to difficulties of recruitment of this population and also due to the limited availability of the cream cheeses considering available VALOBAB ingredient shelf life, which did not allow to extend inclusion period. The 4 volunteers performed the entire protocol, were followed-up and had their data analysed (supplementary figure S2B).

The 4 included participants (1 man and 3 women) had undergone a colectomy because of ulcerative colitis (n=1) or pure colonic Crohn disease (n=3) at least 4 months before the protocol, according to inclusion criteria. They were considered to have healthy small bowels with well-functioning ileostomies (ileal resection $\leq$ 8 cm). Main exclusion criteria included dairy products intolerance, treatment interfering with lipid metabolism, serum cholesterol >7 mmol/L and serum triacylglycerol >3 mmol/L. Individuals taking medication known to interfere with lipid metabolism, having an evolving pathology other than related to their ileostomy (diabetes, hypertension, cardiac, renal or hepatic) or with a psychological illness were not included in the study. In addition, subjects were required not to have made a blood donation for 2 months before the start of the study. Subjects of the VALOBAB-D study were recruited through the digestive surgeons and gastroenterologists of university hospitals of Lyon and Clermont-Ferrand. All participants received written and oral information and their

medical history was reviewed. In addition, they underwent a physical examination and fasting clinical analyses were performed before enrolment.

Allocation group and blinding

In the VALOBAB-C study, we stratified the group allocation on centre and performed the randomization using random number generator of the RAND function from SAS/STAT Software (SAS v9.4, SAS Institute Inc., Cary, NC, USA). Sequences of meal allocation in the VALOBAB-D study were randomized using the same randomization procedure as aforementioned, based on random number generator (RAND function from SAS/STAT Software). For both trials, the anonymization of subjects was performed in each centre using a number corresponding to randomization sequence order. Both volunteers and investigators were kept blind regarding group allocation. To prevent results guided interpretation and subsequent analyses, the biostatistician performed a first set of analyses using colour codes (green, yellow and blue) rather than explicit group allocation (0 g, 3 g and 5 g, respectively), group being considered as categorical factor.

Study products

The cream cheeses were the same for both trials and were based on a buttermilk concentrate rich in milk PL (>30% in dry matter) prepared according to Gassi *et al.* (Gassi, Blot et al. 2016) with a slight modification by ACTALIA *Produits Laitiers* (use of polymer spiral filtration membranes rather than ceramic tubular membranes). Candia (Quimper, France) kindly provided buttermilk from the production of anhydrous milk fat (or so-called butterserum), which is the type of buttermilk presenting the highest milk PL concentration compared to buttermilk from butter production (Bourlieu, Cheillan et al. 2018). It was

concentrated in PL using ultrafiltration at the certified food platform of ENILIA (Surgères, France). The final buttermilk concentrate, with 34 g of milk PL per 100 g of dry matter, was freeze-dried and then incorporated into cream cheese in various amounts to reach 3 g or 5 g of PL per 100 g of cream cheese. The objective was to formulate 3 test cream cheeses with the same total lipid, protein and carbohydrate content, therefore TAGs were substituted by MPL in enriched products. In order to reach the same final protein content, whey proteins were incorporated in the Control (devoid of PL) and 3g-PL cheeses.

Cream cheese production had to supply all the participants of both clinical studies. Given that cream cheeses had a shelf life of 5 months, 5 batches of production were needed over trial duration. The nutritional composition of the test cream cheeses resulting from these different batches is presented in supplementary table S1: for 100 g of cream cheese, the total fat content was 13 g with increased proportions of milk PL. Cheese composition analysis was performed according to the standard methods in the field (available upon request).

Assessment of dietary intake and compliance (VALOBAB-C study)

Subjects recorded their food consumption (study products and other items) for 4 days twice during the study period: at week 1 (run-in period) and at week 5 (last week of the intervention period). A dietician analysed the dietary records using the MICRO 6 diet analyser software (version 6.0; GENI, Villers-Les-Nancy, France). The daily energy intake and the proportion of energy intake from different nutrient sources were calculated. Compliance to each dietary intervention was assessed by counting cheese containers that were returned by volunteers to study coordinators. The mean compliance to dietary intervention was high (>98 %) and similar between groups ($p>0.3$).

Metabolic explorations

All participants came to the nutrition research centre after an overnight of fast and were subjected to repetitive metabolic explorations during each visit. In the VALOBAB-C study, participants were served a standardized breakfast and a lunch including the test cream cheese four hours later (supplementary figure S1C), whereas in the VALOBAB-D study participants had a breakfast including the test cream cheese and a standardized snack four hours later (supplementary figure S2C). In both trials, the visits were divided in two postprandial phases including a first period of 4h post-breakfast in the morning (0 to 240 min) and a second period of 4h post-lunch or post-snack in the afternoon (240 to 480 min).

Blood sampling

Blood samples were obtained at baseline and at fixed intervals after breakfast ingestion from an antecubital arm vein through a catheter and were collected in vacutainer sterile tubes (with EDTA when necessary). Serum and plasma were separated by centrifugation (1500 *g*, 10 min, 4°C) and stored at -20°C or -80°C until further analyses. Some plasma samples were also stored at 4°C for separation of the chylomicron-rich fraction (see below).

Anthropometric and blood pressure measurements

Subjects were weighed to the nearest 0.1 kg (electronic flat scale, SECA) in light clothing and in the fasted state. Subjects also had their height measured to the nearest 0.5 cm by a wall-mounted stadiometer. Body composition was evaluated in all subjects by bioelectrical impedance analysis with a QuadScan4000 (BODYSTAT®, United-Kingdom) to determine lean and fat body mass. Waist circumference was measured using a flexible measuring tape at the point of the navel after an exhalation. Systolic and diastolic blood pressure was measured with a Dinamap Vital Signs Monitor after the subject had been lying down for a least 3 min.

Indirect calorimetry and breath test

Measures of indirect calorimetry were achieved using a QUARK RMR (Cosmed, Rome, Italy). Only indirect calorimetry data from Lyon centre's volunteers are presented because of unfortunate technical issues in the second centre. Respiratory exchanges (VO2 and VCO2) were recorded for periods of 30 or 60 minutes at fasting and during the 8h test period. Substrate oxidations were calculated using Ferrannini's equations (Ferrannini 1988). Urine was collected at 0, 240 and 480 min to determine nitrogen excretion for oxidation calculations. For the hydrogen breath test in the VALOBAB-D study, expired gas samples were obtained at baseline, every 1h for 8h and then at 12h and 24h to check the return to baseline.

Faeces and ileal effluent collection

In the VALOBAB-C study, participants had to collect their stools during the last 20h before each visit V1 and V2. In the VALOBAB-D study, ileal effluents were collected during postprandial periods of the testing days. Just before breakfast, content of the ileostomy bag was emptied. After the breakfast and during the following 8h, cumulated ileal effluents were collected at 2-hour intervals. A sample of the overnight ileal effluent was collected by participants and brought to the centre the morning of each testing. Each fraction of effluents was stored immediately at -20°C before being freeze-dried.

Test meals

VALOBAB-C

The standardized breakfast was a high-fat high-carbohydrate meal and consisted in 2 croissants, chocolate/hazelnut spread (30 g), marmalade (30 g) and coffee or tea (250 mL)

(supplementary table S2). This breakfast provided 571 kcal with 47.8%, 47.0% and 5.2% of energy as lipids (30.3 g), carbohydrates (67.1 g) and proteins (7.4 g) respectively. A lunch was served 4 hours after breakfast containing cooked fish (100 g), pastas (200 g), bread (50 g), compote (100 g) and including the test cream cheese (100 g) with 0, 3 g or 5 g of PL (supplementary table S2). This second meal provided 767 kcal with 24.9%, 51.8% and 23.3% of energy as lipids (21.2 g), carbohydrates (99.2 g) and proteins (44.7 g), respectively. All subjects were given 10 minutes to eat breakfast and 20 minutes for lunch. During each visit, participants were allowed to drink 500 mL of water apart from meal durations.

VALOBAB-D

The test breakfasts were isoenergetic (458 kcal) and equal in nutrient composition (supplementary table S2). They consisted in bread (50 g), butter (10 g), jam (30 g), and the test cream cheese (100 g) containing 0, 3 or 5 g of PL. Moreover, 300 mg of [1,1,1-$^{13}C_3$]-triolein (99 atom% ^{13}C; Eurisotop) and 45 mg of [2,2,3,4,4,6 -D_6]- cholesterol (97-98 atom% ^{2}D; Eurisotop) were added to a portion of the cream cheese. Briefly, tracers were heated together in a Teflon crucible at 70°C for 10 min. A small portion of cream cheese was then added to the crucible and homogenized with a spoon. This labelled mixture was spread on a slice of bread and some additional cream cheese was added on top. Volunteers were instructed to lick the crucible and the spoon in order to minimize incomplete ingestion of tracers. A standardized snack was served 4 hour after breakfast containing pasta, bread, one compote and two skimmed yogurts (0% fat; supplementary table S2). This snack provided 543 kcal with only 3.5% of total energy from lipids, therefore poorly interfering with the digestion of the test breakfast's lipids.

BIOLOGICAL AND BIOCHEMICAL ANALYSES

Blood assays

Total-cholesterol, LDL-cholesterol, HDL-cholesterol, TAG and glucose concentrations were assessed in the serum. ApoB and ApoA1 levels were determined in the plasma. Serum glycerol was analysed and subtracted to the apparent serum TAG concentration in order to obtain the real serum TAG concentration. Such analyses were performed using the automated system Konelab 20 Analyser (Thermo Electron SA, Cergy-Pontoise, France).

Serum insulin and plasma ApoB48 concentrations were quantified according to the manufacturer's instructions by commercially available enzyme-linked immunosorbent assay (ELISA) kits, using, Alpco Insulin Elisa kit (Eurobio, Courtaboeuf, France) and BioVendor R&D ApoB48 Elisa kit (Euromedex Souffelweyersheim, France), respectively. PCSK9 was measured using CircuLex Human PCSK9 ELISA Kit-96 Assays (CliniSciences, France).

Isolation and analysis of chylomicron-rich fractions (CMRF)

CMRF containing chylomicrons and their large remnants were collected by ultracentrifugation as described previously (Vors, Pineau et al. 2013). TAG and cholesterol concentrations of CMRF were measured with a lipase glycerokinase and a cholesterol esterase/oxidase method, respectively, on a Pentra 400 ABX® (Montpellier, France). Hydrodynamic diameter of CMRF was measured by dynamic light scattering at 25°C using a ZetaSizer NanoS (Malvern, UK) using 1.0658 cP and 1.33 as viscosity and refractive index of the aqueous phase, respectively.

Kinetic variables

We calculated the area under curve (AUC) and incremental AUC (iAUC) by the conventional trapezoid rule and maximum postprandial concentration and diameter (c_{max}, d_{max}) for plasma TAG, total cholesterol, ApoA1, ApoB, ApoB48 and CMRF parameters.

^{13}C-fatty acids in plasma lipids and expired CO_2 – ^{2}H-cholesterol in plasma and CMRFs

Sample processing

Internal standards were added according to the fraction analysed (heptadecanoic acid for ^{13}C-FA in total lipids; ergosterol for ^{2}H cholesterol analyses, both from Sigma-Aldrich).

Plasma processing

Plasma samples were submitted to direct methylation as described previously (Vors, Pineau et al. 2013).

Free cholesterol processing

Total lipids were extracted from plasma aliquots with 3 mL of a mixture of chloroform/methanol (2:1, vol:vol) according to the Folch method (Folch, Lees et al. 1957). Free cholesterol fractions were obtained by thin-layer chromatography on silica-gel plates with a mobile phase of hexane: diethyl ether:acetic acid (80:20:1, vol:vol:vol). Free cholesterol was submitted to acetylation according to a modification of the technique of Pouteau (Pouteau, Piguet-Welsch et al. 2003). Briefly, the purified free cholesterol was derivatized with acetic anhydride (150µL) and pyridine (40µL) overnight at room temperature.

CMRF processing

CMRF were isolated as described previously (Vors, Pineau et al. 2013). Lipids were extracted from CMRF according to the Folch method (Folch, Lees et al. 1957). Free cholesterol fraction was then processed, as mentioned above for the plasma free cholesterol fraction.

Of note, both free cholesterol and cholesteryl esters were analysed from thin layer chromatography plates from the postprandial plasma and CMRF samples of 3 different tests/meals of one subject. ^{13}C-cholesterol was homogeneously present in both forms (half in free form, half in esterified form) and results were similar for both forms (i.e., much less tracer present in the fraction after PL-enriched meals vs Control meal); therefore, we carried on with the analysis of the free ^{13}C-cholesterol in plasma and CMRF for all subjects.

Sample analysis

The amount and isotopic enrichment of oleic acid in plasma were determined by using gas chromatography–combustion isotope ratio mass spectrometry (Isoprime, Elementar UK Ltd) (Vors, Pineau et al. 2013). The capillary column was a SP2380, 30 m× 0.25 mm× 0.20 μm film thickness (Supelco). ^{13}C isotopic enrichment was expressed in δ ‰ against Pee Dee Belemnite ^{13}C international standard (PDB) and then converted into Mole Percent Excess (MPE). The amount and isotopic enrichment of free cholesterol in plasma and CMRF were assessed by gas chromatography–pyrolysis isotope ratio mass spectrometry (Delta V; Thermo Scientific). The capillary column was a VF 5-MS, 25 m× 0.25 mm× 0.25 μm film thickness (Agilent). Helium was used as the carrier gas. Injection (1 μL) was performed in splitless mode at 280 °C. Cholesterol acetate was separated at constant flow (1.1 mL.min^{-1}) with the following oven program: (a) 100 °C for 1 min; (b) increase at a rate of 20 °C.min^{-1} to 290 °C; (c) hold at 290 °C for 7 min. A post-run program was used: 2.5min at 310°C with a constant flow of 1.5 mL.min^{-1}. ^{2}H isotopic enrichment was expressed in δ ‰ against SMOW (standard mean ocean water, ^{2}H international standard) and further transferred into MPE.

Calculations of exogenous lipid oxidation from indirect calorimetry and breath tests

Exogenous lipid oxidation was calculated according to Binnert *et al.* from data of indirect calorimetry and breath tests (Binnert, Pachiaudi et al. 1998). Here the formula was adapted to the use of one labeled triglyceride (^{13}C triolein) in the VALOBAB-D study:

Exogenous lipid oxidation (% of ingested fat) =

$$\frac{\left\{\left[\left\{\left[AP\ CO_2\ (t) + AP\ CO_2\ (t_{-30})\right]/2\right\} - AP\ CO_2\ (t_0)\right]/100\right\} \times \dot{V}\ CO_2}{(A) \times 22.4 \times dARF} \times 100 \times \Delta t$$

With: $(A) = \left\{\left[\ AP\ {}^{13}TG\ C18:1\ \right]/100\right\} \times \left[(0.30/888.40) \times 57\right]$

Where: AP CO_2 (t) is the AP value of the expired CO_2 at time t, AP CO_2 (t_0) is the AP value of the expired CO_2 at time t_0, AP tracers is the calculated AP value of the labelled mixture of TAG (tracers) and $\dot{V}\,CO_2$ is the production rate of expired CO_2 (indirect calorimetry), Δt is the period of measure in minutes. Mean molecular weight of triolein is 888.40 g/mol. Mean number of carbons in triolein is 57. dARF (Acetate Recovery Factor) is the correction factor for incomplete recovery of ^{13}C bicarbonate (0.505 for normal-weight subjects) (Antoun, Momken et al. 2010) and 22.4 is the volume (L) of a mole of CO_2. The 8h-cumulative exogenous lipid oxidation is obtained as the sum of exogenous lipid oxidation calculated for each period of measure.

Faecal microbiota and SCFA analyses

Quantitative PCR analysis of faecal bacterial communities.

Faecal samples from each subject were collected at V1 and V2 (before and after the 28 days period of supplementation) and kept frozen at -80°C. Population levels of the main phylogenetic groups and bacterial species of gut microbiota were quantified using real-time PCR (Eppendorf Master Cycler ep RealPlexTM 2.0, Hamburg, Germany). Total DNA from faecal materials (200 mg) was extracted using the QIAmp DNA Stool Mini Kit (Qiagen Inc., Mississauga, ON, Canada) after mechanical disruption of cells. Extracted DNA was then used to amplify regions of the 16S rDNA genes with specific primers for different bacterial groups or species. The real time quantitative PCR was performed with the Brilliant SYBR green system (Roche, Manheim, Germany) with specific primers and in the conditions previously described for Eubacteria (Furet, Firmesse et al. 2009), *Bacteroides-Prevotella* group (Furet, Firmesse et al. 2009), *Firmicutes* (Guo, Xia et al. 2008), *C. leptum* group (Crouzet, Gaultier et al. 2013), *C. coccoides* group (Crouzet, Gaultier et al. 2013), *Roseburia - E. rectale* group (Crouzet, Gaultier et al. 2013), *Enterobacteriaceae* (Castillo, Martin-Orue et al. 2006), *Bifidobacterium spp.* (Delroisse, Boulvin et al. 2008), *Lactobacillus-Leuconostoc-Pediococcus* (Furet, Firmesse et al. 2009), *Veillonella spp.* (Tana, Umesaki et al. 2010), *Faecalibacterium prausnitzii* (Crouzet, Gaultier et al. 2013), *Akkermancia muciniphila* (Collado, Derrien et al. 2007), *Bilophila wadworthia* (McOrist, Warhurst et al. 2001) and *Escherichia coli* (Huijsdens, Linskens et al. 2002).

The number of bacteria was expressed as [log of 16S rDNA gene copy per gram of faeces]. Impact of the intervention was calculated as the difference (V2-V1) in [log of 16S rDNA gene copy per gram of faeces], corresponding to the log of the fold-change (V2/V1) of [16S rDNA gene copy per gram of faeces].

Short Chain Fatty Acids (SCFA) analysis

SCFA were quantified in faecal water by gas phase chromatography (Clarus 580 GC, Perkin Elmer, USA). Faecal water was obtained from 1g of each faecal sample collected in Centre 2 (Clermont-Ferrand) kept frozen at -20°C, following the method described by (Zhao, Nyman et al. 2006).

Faecal loss assessment

Coprostanol and cholesterol measurements

Regarding the VALOBAB-C study, measurements of coprostanol and cholesterol contents were performed on stools collected at V1 and V2 in Centre 1 (Lyon). Stools were weighed and lipids extraction was realized on 500 mg of homogenized stools. Lipids contained in stools were extracted by modified Folch reagent (methanol/chloroform mix, 1:1, v/v). Tocopherol acetate was used as an internal standard and added to homogenized stools before lipids extraction. Lipid extracts were dried under nitrogen. The dry residue was re-dissolved in chloroform and washed with a saline solution to remove remnant proteins. After liquid-liquid extraction, the chloroform phase was recovered and then concentrated under nitrogen. The dry residue was re-dissolved in chloroform/methanol (2:1, v/v) and lipids classes were separated by thin-layer chromatography on silicagel plate using petroleum ether, ethylic ether and acetic acid (85:13:2, v/v/v) as mobile phase. The plates were sprayed with bromophenol blue and individual bands of cholesterol, coprostanol and tocopherol acetate were scraped off in a same tube. Only bands of free sterols were detectable. This mix of fractions was dissolved into methanol/hexane (1:3, v/v). Hexane phase was recovered and concentrated under nitrogen. Dry residue was dissolved in hexafluoro-1,1,1,3,3,3-propanol-2 and trifluoroacetic anhydride (1:2, v/v) to allow its derivatization during 30 minutes at 37°C.

Derivatized sample was then dried under nitrogen and dissolved in dichloromethane before their injection into gas chromatography system. Gas chromatography system (GC Focus, ThermoFisherScientific, Les Ulis, France) was equipped with a DB-5MS capillary column 30 m x 0.25 mm 0.25µm (Agilent, Les Ulis, France). Helium was used as the carrier gas. The initial ramp temperature began at 120°C for one minute and then temperatures were programmed as follows: 5°C/min until 220°C maintained for 20 minutes, 20°C/min until 260°C maintained for 8 minutes, 1.5°C/min until 290°C maintained for 5 minutes allowing separation of cholesterol, coprostanol and tocopherol acetate fractions. Identification of coprostanol, cholesterol and tocopherol acetate was made by frequent comparison of their retention times with those of standards. Amounts of coprostanol or cholesterol were determined using ChromCard software (ThermoFisherScientific, Les Ulis, France) as the ratio of the area of coprostanol or cholesterol multiplied by the amount of internal standard multiplied by the response factor on the area of internal standard. These results were based on the amount of stools and expressed as µg of coprostanol or cholesterol per g of stools.

Total lipid content

Regarding the VALOBAB-C study, measurement of total lipid content was performed by near-infrared reflectance analysis (FENIR 8820, Perten, Hamburg, Germany) in homogenized stools collected in Centre 1 (Lyon) at V1 and V2. The results expressed in percentage of total lipid content were related to the weight of each stool and expressed in g per 24h.

Ileal lipid analysis

Total lipids

Total lipids of lyophilized samples (100 mg) were extracted by homogenization with chloroform–methanol (2:1, v/v) according to the Folch method (Folch, Lees et al. 1957). After

drying under nitrogen, total lipids were determined gravimetrically and were dissolved precisely with 1 mL of chloroform/methanol (2:1). This stock solution of total lipids was stored at -20°C.

Sphingomyelin content

SM was separated from other PL classes by HPLC coupled to an evaporative light-scattering detector (HPLC-ELSD) (Becart 1990; Rombaut, Camp et al. 2005). The different classes of phospholipids were separated using a silica normal-phase column (Lichrospher Si 60, 3 µm, 100 x 4.6 mm, Waters). The chromatographic separation was carried out using a linear binary gradient according to the following scheme: t0 min 90%A, 10%B 0%C; t20min 42%A 52%B 6%C; t30min 32%A 52%B 16%C; t55min 30%A 70%B 0%C; t60min 90%A 10%B 0%C. Total chromatographic run time was 75 min per sample, which consisted of a 60 min analysis and 15 min to restore initial conditions and re-equilibration. Eluent A consisted of hexane-tetrahydrofuran (99:1, v/v), eluent B of isopropanol-chloroform (80:20, v/v/v) and eluent C of isopropanol–water (50:50, v/v/v). The flow rate of the eluent was 1.0 ml/min. Identification of SM was carried out by comparison with the retention time of pure standard (Avanti polar Lipids, USA). Calibration curves for each compound were calculated from the area values of stock solution of pure standards between 0,05 to 1 mg/mL. Results were expressed as mg of SM per g sample.

Sphingomyelin species profile

For SM species profiling by electrospray ionization-tandem mass spectrometry (ESI-MS/MS), SM were extracted in chloroform:methanol (1:2 v/v) according to the method by (Kyrklund 1987) in the presence of N-palmitoyl(d31)-D-*erythro*-sphingosylphosphorylcholine (C16:0D31SM) from Avanti Polar Lipids. Sphingolipids were isolated by a step of saponification, fractionated and desalted using reverse-phase Bond Elut C18 columns. The

dry extracts were kept at -20°C until tandem mass spectrometry analysis. Samples were homogenized in chloroform:methanol (1:2 v/v) and analysed by direct flow injection on an triple-quadrupole mass spectrometer (API 4500 QTRAP MS/MS; Sciex Applied Biosystems, Toronto, Canada). SM species were measured in the positive ionization mode using the multiple reaction monitoring (MRM) method at a flow rate of 200µl/min (analysis time of 3 minutes). The quantity of each molecular specie was calculated from the ratio of its signal to that of the corresponding internal standard and normalized to the amount of lyophilized sample.

Cholesterol content

Cholesterol (sum of total and esters) was analysed on a FOCUS GC gas chromatograph (Thermo Electron Corporation) equipped with an online injector and a flame ionization detector. Two hundred µL of total lipids were dried under nitrogen and then sylilated using n-methyl imidazole / MSHFBA (1/20). Internal standards (5β-cholestanol and stigmastadiene for sterols) (Sigma Aldrich) were added before sylilation to assume identification and quantitate each compound. Separation of lipids was performed with a ZB5-fused silica capillary column (30m length × 0.25 mm internal diameter, 0.25 µm film thickness; SGE, Courtaboeuf, France). Hydrogen was used as the gas vector (constant flow, 1.5mL/min). The injector and detector temperatures were at 300°C and 340°C, respectively. The oven temperature was set to 240°C, increased to 255°C at a rate of 4°C/min with a 31-min hold, increased to 320°C at a rate of 50°C/min and then left at this temperature for 1 min.

Primary outcomes and sample size calculation

VALOBAB-C

The primary outcome was the effect over 4 weeks of the daily consumption of MPL (1 control group and 2 increasing doses) on fasting serum concentration of total cholesterol, defined as the difference between V1 and V2. Secondary outcomes included the same effect on plasma concentration of other lipid risk factors (TAG, LDL-cholesterol, HDL-cholesterol, ApoB) and postprandial lipid metabolism, again measured between V1 and V2. Despite our design planned longitudinal analyses with multiple time points measures, sample size calculation could only rely on literature with single time point information and the expected effect (mean±standard deviation (SD)) was derived from Keller *et al.* (Keller, Malarski et al. 2013). Effect on fasting concentration of total cholesterol was expected to be 0.0±0.6 mM in the Control group, -0.5±0.6 mM in the 3g-PL group and -1.0±0.6 mM in the 5g-PL group. Setting type I error at 0.05 and using double sided tests, all the tests on contrasts between groups (including global "MPL" effect as binary factor, lumping both 3g and 5g doses *vs* Control), performed through a general linear model, had a statistical power over 0.84 including 26 subjects per group. Thus, the minimum total sample size was set to 78 subjects.

As stated in previous sections (see Study participants), the total sample size was 58. Regarding longitudinal serum concentration of total cholesterol over the 10 postprandial times (from 0 to 480 min), we estimated actual power accounting for our longitudinal design analysis. For a total sample size of 58, the probability to show significant group effect turned out to be over 0.999 basing on actual mean concentrations and common standard deviation of 0.42 mM.

VALOBAB-D

The primary outcome was the effect of milk PL content on ileal sphingomyelin (SM) output. The secondary outcomes were the effect of milk PL content on postprandial lipid metabolism (plasma lipids and isotopic tracers). A previous study on the impact of increasing doses of milk SM on SM concentrations in human ileostomy content of 6 subjects was used for the sample size calculation (Ohlsson, Hertervig et al. 2010). Based on these results, the expected ileal SM outputs were of 2.5±0.7 µg/g, 6.7±1.9 µg/g and 13.4±3.9 µg/g, for 0g, 3g and 5g of milk PL, respectively. Assuming unfavourable situation of a parallel group design and the highest variability (i.e. common SD at 3.9 µg/g), a total sample size of 12 subjects was calculated to reach minimum power of 0.86 in detecting significant changes in SM ileal output between the 3 meals. We expected the present crossover design to decrease variability of outcomes and thus enhance power analysis.

STATISTICAL ANALYSES

VALOBAB-C

We described continuous variables as mean±standard error (SEM) and, where appropriate, i.e. for distribution that clearly deviate from normality, we displayed median and interquartile range. All data were analysed (no exclusion of subjects' data). In case of missing data, we did not apply any imputation method. For each parameter, we computed the difference between visits (V2-V1) and used it as response variable in the models. The design of our study planned to measure each parameter longitudinally at each visit (as aforementioned in previous sections), with number of time points ranging from 1 (single time point) up to 10. Apart from single time point parameters that were analysed through general linear model and subsequent post-hoc tests following Tukey, we performed mixed linear modelling (MIXED procedure

from SAS/STAT Software) to account for within-subject repeated measures, seeking for main effects, i.e. at least "group" effect, time effect and interaction between these 2 factors. In order to check for any confounding effect, these analyses were also carried-out adjusting for centre, age and waist circumference quartiles. Post-hoc analyses were performed following Tukey-Kramer to both detail main effects and control for familywise type I error. In case of residual distribution departing from normality, the analyses were performed on ranks and the results were displayed using median and interquartile range. Visit effect, i.e. intra-group comparisons between first and second visit, was available for each group testing mean difference against 0. In addition, we performed all the analyses considering global "milk PL" effect as binary factor, i.e. lumping together 3g-PL and 5g-PL doses in one group *vs* Control. All analyses were performed on SAS v9.4 for windows (SAS Institute Inc. Cary, NC, USA) with a type I error set at 0.05.

VALOBAB-D

Each subject served as his own control (crossover design). All data are presented as means ± SEM and were analysed with GraphPad Prism 7 software. A Shapiro-Wilk test was performed to determine normality of data. For normally distributed data, repeated measures 1-way ANOVA (according to meal) or 2-way ANOVA (according to meal and time) were performed followed by Tukey's post-hoc tests. For non-normal data (plasma iAUC of [^{2}H]-cholesterol), a Friedman test was performed followed by Dunn's post-hoc tests. For each parameter a Mann-Whitney test was also performed to compare the Control meal to PL meals, i.e. with data of the 3g-PL and 5g-PL together within the PL meals.

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