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Impact of Dietary Dairy Polar Lipids on Lipid Metabolism of Mice Fed a High-Fat Diet

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Supporting Information

ABSTRACT: The effect of milk polar lipids on lipid metabolism of liver, adipose tissue, and brain and on composition of intestinal microbiota was investigated. C57BL/6J mice were fed a high-fat diet (HFD) for 5 weeks, followed by 5 weeks with HFD without (control) or supplemented with total polar lipids (TPL), phospholipids (PL), or sphingolipids (SPL). Animals fed SPL showed a tendency for lower triglyceride synthesis ($P = 0.058$) in the liver, but not in adipose tissue. PL and TPL reduced de novo hepatic fatty acid biosynthesis. The ratio of palmitoleic to palmitic acid in the liver was lower for animals fed SPL or TPL compared to control. There was little effect of the supplementation on the cecal microbiota composition. In the brain, DHA (C22:6) content correlated negatively with tetracosanoic acid (C24:0) after TPL supplementation (−0.71, $P = 0.02$) but not in control (0.26, $P = 0.44$). Arachidonic acid (C20:4) was negatively correlated with C24:0 in both groups (TPL, −0.77, $P = 0.008$; control, −0.81, $P = 0.003$).

KEYWORDS: *dairy polar lipids, phospholipids, sphingolipids, diet-induced obesity, brain lipid metabolism, cecal microbiota, metabolic syndrome, mass isotopomer distribution analysis*

INTRODUCTION

Food intake and composition in affluent countries have been significantly affected by extraordinary technological advances in food processing during the past decades. Dietary habits shifted toward higher intakes of calories, primarily from saturated fats and *trans*-fatty acids,[1] which have been associated with an increased prevalence of metabolic disorders, such as diabetes, cardiovascular disease, and nonalcoholic fatty liver disease.[2] Moreover, such dietary-induced metabolic dysfunctions have recently been shown to be capable of disrupting brain homeostasis and impairing neurogenesis.[3,4] Although certain genetic characteristics may increase an individual's susceptibility to obesity and associated metabolic disturbances, the use of dietary supplements could help to ameliorate the effects of these disturbances. Therefore, the discovery of new ingredients with these functionalities has been gaining importance.

Bovine milk polar lipids, such as phospholipids, sphingolipids, and glycolipids, constitute up to 0.04% of raw milk content. During milk processing these polar lipids are preferentially distributed to the aqueous phase. Products such as buttermilk and butterserum have a high level of polar lipids, which can reach 2 and 11% of the dry matter, respectively.[5] These products are considered to be low-value byproducts of the dairy industry and currently have a limited market; however, they are increasingly recognized as a source of functional and nutritional ingredients.[5,6]

Recent studies indicate that dietary polar lipid extracts (i.e., sphingolipids and/or phospholipids) may mitigate the undesirable effects of high-fat diets. For example, phytosphingosine (a sphingolipid derivative) has been shown to reduce plasma cholesterol and triacylglycerol content in the liver,[7] and soy phospholipids are known to prevent the development of steatosis in rats.[8] Wat and colleagues[9] observed a significant reduction in hepatomegaly, hyperlipidemia, and liver steatosis in mice fed a high-fat diet supplemented with milk phospholipids, but not in mice fed a normal chow diet supplemented with the same phospholipid-rich extract. The mechanism involved in the ability of polar lipids to ameliorate the impact of high-fat diet is not fully understood, but may be related to the inhibition of intestinal fat absorption[7] and/or

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reduction of expression of enzymes affecting fatty acid synthesis.[8,9]

High intakes of saturated fatty acids have also been associated with adverse effects on brain homeostasis through the development of chronic low-grade inflammation[10−13] and contribute to neurodegenerative diseases by impairing hippocampal synaptic plasticity.[14,15] Some exogenous phospholipids induce anti-inflammatory signals ameliorating increased levels of pro-inflammatory cytokines. For example, phosphatidylserine was able to prevent lipopolysaccharide-induced effects and inhibited microglial activation.[16] Phosphatidylserine/phosphatidylcholine liposomes were reported to inhibit the production of tumor necrosis factor-α induced by amyloid β and interferon-γ.[17] In addition, phosphatidylserine liposomes were able to inhibit inflammation of the central nervous system and improve the survival rate of neurons after ischemia−reperfusion injury.[18] High levels of pro-inflammatory cytokines have been reported in the hypothalamus of rats fed high-fat diets,[10,19] and these may activate phospholipase A_2 isoforms that affect lipid metabolism in the brain.[20,21] Therefore, we hypothesized that the supplementation of dietary phospholipids such as phosphatidylcholine and phosphatidylserine can attenuate the effect of a high-fat diet in brain lipid metabolism.

Environmental factors, such as the metabolism of intestinal microbes, have also been proposed to contribute to the development of metabolic disorders.[22] Because the digestion of sphingolipids and phospholipids in the gut is slow and incomplete, the colon and intestinal microbiota are exposed to nondigested phospholipids[23] and sphingomyelin.[24] Taken together, this suggests that the metabolism of some polar lipids by the intestinal microbes has potential implications for host metabolism.

In the present work we investigate if milk-derived phospholipids and/or sphingolipids would reduce the effects of a high-fat diet in young mice. A heavy water labeling experiment was used to identify the effect of bovine milk polar lipids in the lipogenesis in the liver and adipose tissue. Fatty acids and lipid profiles were analyzed to investigate changes in the lipid metabolism in the brain. The composition of the cecal microbiota was assessed by pyrosequencing of 16S rRNA genes. We evaluated three milk polar lipid fractions (1, phospholipids (PL); 2, sphingolipids (SPL); and 3, polar lipids (TPL), which was a mixture of phospholipids and sphingolipids with the following aims: 1, investigate which of the fractions are active in the liver lipogenesis; 2, determine whether any biological activity would help to mitigate effects resulting from a high-fat diet during the first 5 weeks; 3, evaluate whether any biological activity could also be observed in the adipose tissue; 4, determine whether it would have any effect in lipid metabolism of mouse brain compared to control; and 5, investigate if milk polar lipids would affect the cecal microbiota.

MATERIALS AND METHODS

Chemicals and Materials. Thin layer chromatography (TLC) silica gel 60 precoated in aluminum sheets were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Silica gel for flash chromatography (230−400 mesh) and all solvents were purchased from Thermo Fisher Scientific (Auckland, New Zealand). TLC, 3 N methanolic hydrochloric acid, L-α-phosphatidylinositol ammonium salt solution (from bovine liver approximately 98%), L-α-phosphatidyl-L-serine (from *Glycine max* (soybean)), L-α-phosphatidylcholine (from soybean), and sphingomyelin (bovine brain) were purchased from Sigma-Aldrich. Ammonium hydroxide, acetic acid, and triethylamine were of analytical grade, and deuterated water and glycerol-d_5 were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). 1-Palmitoyl-2-oleoyl-*sn*-phosphatidylethanolamine was purchased from Avanti Polar Lipids (Alabaster, AL, USA). A commercial β serum powder was sourced from Tatua Co-Operative Dairy Co. Ltd. (Morrinsville, New Zealand).

Extraction and Purification of Phospholipids. Beta-Serum (1 kg) was mixed with ethanol (2.5 L). The mixture was heated to 40 °C and stirred by mechanical agitation for 30 min. The mixture was then filtered under vacuum using a glass fiber filter. The resulting retentate was re-extracted using the same procedure described above. The resulting filtrates were combined, and the solvent was evaporated under vacuum at 40 °C to yield the total fat extract. The whole procedure was repeated five times, resulting in the extraction of 320 g of total fat extract. TPL were obtained from the total fat extract as described elsewhere.[25] Part of this TPL extract was used as the dietary supplement in this study, and the remaining amount was used to obtain the PL extract and SPL extract. The TPL extract (20 g) was dissolved in 100 mL of chloroform/methanol (2:1 v/v), to which 10 g of silica gel was added. The solvent was removed, and the mixture was applied to a chromatographic column (7 cm × 30 cm glass tube) containing 300 g of silica gel. The column was initially eluted with a solvent mixture (65:25:4:0.1) of chloroform, methanol, water, and ammonium hydroxide (1.9 L), followed by a 20:40:5:0.1 mixture of chloroform, methanol, water, and ammonium hydroxide (3.3 L). The lipid fractions eluted from chromatographic column were monitored by TLC. Fractions containing PL or SPL were combined and evaporated on a rotavapor at 40 °C and then lyophilized. This procedure was repeated four times to yield the amount required (PL extract = 32.7 g and SPL extract = 10.2 g). The amounts of phospholipids and sphingomyelins in the original beta-serum and extracts (Table S1 in the Supporting Information) were quantified using an HPLC-ELSD/Shimadzu (Kyoto, Japan) instrument equipped with two LC-10 Advp pumps, an SCL-10 Advp gradient system, a DGU-14 Advp module degasser, and an automatic injector. The analytical column used was a YMC-pack PVA-SIL-NP column (250 × 4.6 mm, 5 μm). Chromatographic separation was carried out using a linear binary gradient according to the following scheme: t_0 = 0% B, t_{30} 40% B, and isocratic conditions (40% B) for 1 min. Eluent A consisted of chloroform/isopropanol/triethylamine/acetic acid (75:25:0.08:1.0 v/v/v/v) and eluent B of methanol/water/triethylamine/acetic acid (95:5:0.08:1.0 v/v/v/v). The flow rate of the eluent was 1.0 mL/min. An ELSD-LT II Shimadzu model ELSD was used for detection; the pressure of the nebulizer gas (nitrogen) was maintained at 350 kPa, and the drift tube temperature was set at 50 °C. Identification of phospholipids and sphingomyelins was carried out by comparison with the retention time of pure standards. Calibration curves for each compound were calculated from area values obtained by injecting different volumes (1−14 μL) of chloroform/methanol (2:1 v/v) solution containing phosphatidylethanolamine (PE) (3.7 mg/mL), phosphatidylinositol (PI) (1.2 mg/mL), phosphatidylcholine (PC) (7.15 mg/mL), phosphatidylserine (PS) (2.1 mg/mL), and sphingomyelin (SM) (5.4 mg/mL). The concentration of polar lipids in the extracts (TPL, PL, and SPL) is presented in supplementary Table S1 in the Supporting Information. The composition of fatty acids from the diets is shown as supplementary Table S2 in the Supporting Iniformation. The extracts were shipped to Research Diets (New Brunswick, NJ, USA) for the preparation of the diets (Table 1).

Animal Experiment. This study was approved by the AgResearch Grasslands Animal Ethics Committee in Palmerston North (Animal Ethics Committee Application 11958), New Zealand, according to the Animal Protection Act (1960) and Animal Protection Regulations (1987). Fifty-two female C57Bl/6J mice (4 weeks of age) were obtained from the Animal Research Centre (Perth, Australia) and housed under conventional animal laboratory conditions (Ulyatt Reid Facility at AgResearch, Palmerston North, New Zealand). After 1 week of adaptation, the mice were fed a Western-style high-fat diet (a pelleted "western" diet, Research Diets, New Brunswick, NJ, USA) for 5 weeks (Table 2) and had ad libitum access to water.

At week 6 of the study the mice received an intraperitoneal (ip) injection of deuterated water (^2H_2O), 15 μL of ^2H_2O/g body weight,

Table 1. Composition of Dairy Polar Lipids (Percent) Supplemented to the Diets[a]

group	SM	PC	PS	PI	PE
SPL	0.35	0.04			
PL	0.025	0.33	0.07	0.01	0.32
TPL	0.42	0.33	0.15	0.03	0.26

[a]SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine. Group SPL was supplemented with sphingolipid-rich extract, group PL was supplementated with phospholipid-rich extract, and group TPL was supplementated with total polar lipid extract. Average of 5 weeks of feeding of a high-fat diet and with (SPL, PL, and TPL) or without (control) supplements.

Table 2. Composition (Grams per Kilogram) of Diet Produced by Research Diet (New Brunswick, NJ, USA)

	control	SM	PL	TPL
protein	28.0	27.8	26.4	26.3
carbohydrate	47.8	47.6	47.1	47.0
fat	28.0	26.7	26.4	26.3
casein, 80 mesh	233.1	232.1	229.9	229.1
L-cystine	3.5	3.5	3.4	3.4
corn starch	84.8	84.5	83.7	83.4
maltodextrin 10	116.5	116.0	114.9	114.6
sucrose	201.4	200.5	198.6	198.0
cellulose, BW200	58.3	58.0	57.5	57.3
corn oil	29.1	29.0	28.7	28.6
lard	206.9	206.0	204.0	203.3
mineral mix S10026	11.7	11.6	11.5	11.5
dicalcium phosphate	15.1	15.1	14.9	14.9
calcium carbonate	6.4	6.4	6.3	6.3
potassium citrate, 1 H_2O	19.2	19.1	19.0	18.9
vitamin mix V10001	11.7	11.6	11.5	11.5
choline bitartrate	2.3	2.3	2.3	2.3
SM extract		4.2[a]		
PL extract			13.6[a]	
TPL extract				16.9[a]
total	1000.0	1000.0	1000.0	1000.0

[a]The amount of polar lipid extract was adjusted to achieve the percentage of polar lipids shown in Table 1.

to enrich approximately 2.5% of their body water with ^2H_2O.[26] Food was removed 8 h before the injection and returned 2 h after injection to allow for isotope equilibration. Subsequently, the mice were randomly allocated on the basis of body weight to one of four groups (n = 13 mice per treatment): group 1, control group (control) on a high-fat diet; group 2 (TPL), high-fat diet supplemented with TPL extract; group 3 (PL), high-fat diet supplemented with PL extract; group 4 (SPL), fat diet supplemented with SPL extract (Table 2). These diets were fed ad libitum for 5 weeks. During this period mice had ad libitum access to drinking water, which contained 4% of ^2H_2O to determine fractional synthesis of lipids through mass isotopomer distribution analysis (MIDA).

Food intake, body weight, and general health scores were monitored three times per week. Spot urine samples were collected weekly, 8 h before ip injection of ^2H_2O and 2 h after isotope equilibration.

Sample Collection and Storage. At week 11, mice were humanely euthanized using CO_2 asphyxiation followed by cervical dislocation and cardiac puncture to collect a blood sample. To minimize variations between food intake before sacrifice, mice were fasted for 14 h; food was returned for 2 h and then removed again 2 h before they were euthanized in staggered groups.[27] Blood was immediately separated into plasma by centrifugation (2000g, 3 min). The liver was excised, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C. The gastrointestinal tract was removed and the cecum separated to collect contents, which were snap-frozen in liquid nitrogen and stored at −80 °C. Inguinal fat pads were excised and snap-frozen in liquid nitrogen at −80 °C. Whole brains were excised and snap-frozen in liquid nitrogen and stored at −80 °C.

Extraction of Lipids. Lipids were extracted from liver and adipose tissues using a method described by Turner et al.[26] Briefly, the tissues (approximately 200 mg) were blended with 2 mL of methanol/chloroform (2:1), ground until homogeneous, and then centrifuged (2000g) to remove protein. The solution was extracted with 2 mL of each chloroform and water. The aqueous phase was discarded, and the lipid fraction was dried under a nitrogen stream. The lipids from the diet and feces were extracted using a procedure similar to that described for the tissue samples.

The brain samples (∼300 mg) were obtained from the frozen tissues of two groups: the control and TPL groups. During the procedure the metal support holding the sample was kept in dry ice and liquid nitrogen was added to prevent the samples from thawing. The samples were then ground and homogenized in the presence of liquid nitrogen. To this homogenate was added 8 mL of chloroform/methanol 2:1 (v/v), and the samples were filtered (grade 2 Whatman filter paper). Subsequently, 2 mL of chloroform/methanol 2:1 (v/v) was used to wash the grinder, filtered, and added to the first filtered homogenate, which was allowed to separate thoroughly; the chloroform phase was recovered and then dried under a nitrogen stream.

Free Cholesterol. The amount of free cholesterol in the lipid extract of liver was quantified using HPLC-ELSD. The analytical column used was YMC-pack PVA-SIL-NP column (250 × 4.6 × mm i.d., S-5 μm, 12 nm). The chromatographic separation was carried out using a linear binary gradient according to the following scheme: t_0 = 0% B, t_{15} 100% B, and isocratic conditions (100% B) for 5 min. Eluent A consisted of hexane and eluent B of chloroform. The flow rate of the eluent was 1.0 mL/min. An ELSD-LT II Shimadzu model ELSD was used; the pressure of nebulizer gas (N_2) was maintained at 350 kPa, and the drift tube temperature was set at 50 °C. Identification of cholesterol was carried out by comparison with the retention time of pure standards. A calibration curve was calculated from area values obtained by injecting different volumes (5−200 μL) of a chloroform/methanol (2:1 v/v) solution containing free cholesterol (0.75 mg/mL).

Fatty Acid Profile. Lipid extracts from liver and adipose tissue were transesterified by incubation with 3 N methanolic HCl at 55 °C for 60 min. Fatty acid methyl esters were separated from glycerol according to the Folch technique,[28] with the modification that pure water was used for the aqueous phase rather than 5% NaCl as described by Turner et al.[26] The aqueous phase was used for glycerol analysis to perform the calculation of fractional synthesis of lipids through MIDA as described for mass isotopomer distribution analysis. Lipid extracts from the brain were transesterified by incubation with 5% methanolic sulphuric acid at 80 °C for 30 min. Fatty acid methyl esters were analyzed by GC-MS using a Shimadzu QP2010 GC-MS and an RT 2330 capillary column (10 m × 0.18 mm i.d. × 0.1 μm film, Restek). The temperature program was as follows: 50 °C initial hold for 2 min increased to 200 °C at rate of 150 °C/min. The split ratio was 40:1 with a helium flow under pressure of 103 kPa.

Direct Infusion Mass Spectrometry. The lipid extracts from the brain tissue were analyzed by direct infusion electron spray mass spectrometry (ESI-MS). Direct infusion mass spectrometry was performed using a linear ion trap mass spectrometer (LTQ-XL) (Thermo Finnigan, San Jose, CA, USA). Thermo Finnigan Xcalibur software (version 2.07) was used for data acquisition and processing. The samples were diluted 10-fold with acetonitrile containing 0.1% formic acid and were directly infused into the mass spectrometer at 5 μL/min. Samples were run in a random order. The mass spectrometer was set for ESI in both positive and negative modes. The spray voltage was 5 kV and the capillary temperature, 275 °C.

Mass Isotopomer Distribution Analysis. The fractional synthesis of lipids in liver and adipose tissue was calculated using MIDA,

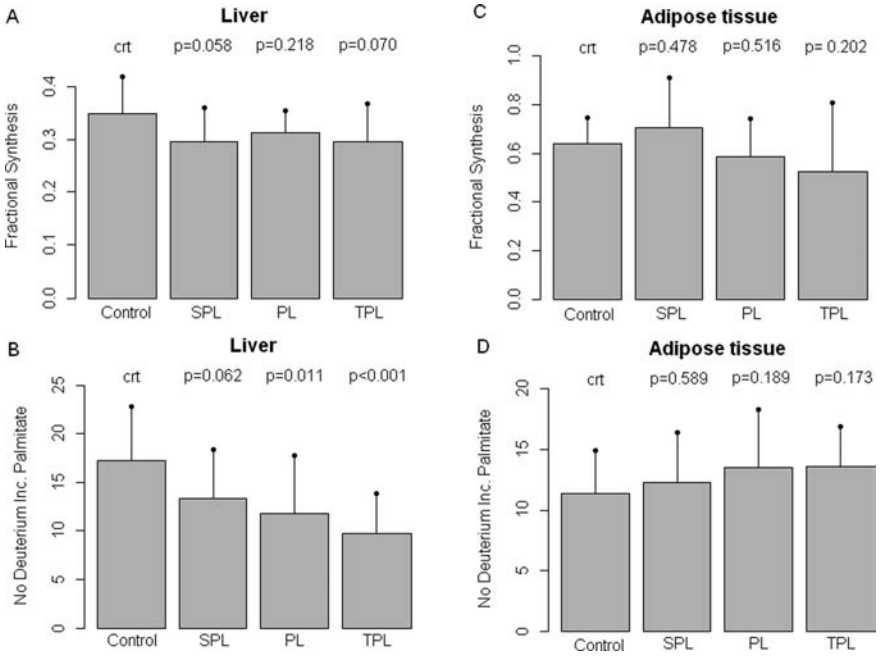

Figure 1. Mass isotopomer distribution analysis on liver and adipose tissue. Total polar lipid extract (TPL), phospholipid-rich extract (PL), and sphingolipid-rich extract (SPL). Treatments were compared to control group using ANOVA, and resulting *P* values are shown above the bars. Fractional synthesis, fractional synthesis of triglycerides; No Deuterium Inc. Palmitate, number of ^{2}H incorporated in the palmitic acid.

which estimates the likelihood to insert deuterium atoms (^{2}H) into the de novo produced lipid of a given pathway(s) in the lipid synthesis. The number of incorporated ^{2}H and the fraction of newly synthesized triglycerides were calculated according to the method of Lee et al.[29] The number of incorporated ^{2}H was also estimated for palmitate (C16:0) in both liver and adipose tissues.

A procedure for glycerol derivatization was developed and validated to allow the use of electron impact ionization instead of chemical ionization in the GC-MS analysis. In this procedure, the aqueous phase containing glycerol obtained from the transesterification step as described above was lyophilized overnight, and glycerol was converted to glycerol tris(trifluoroacetate). Glycerol was converted to glycerol tris(trifluoroacetate) by incubation with 250 μL of dichloromethane, 100 μL of 0.05 M pyridine in dichloromethane, and 10 μL trifluoroacetic anhydride. Tubes were vortexed for 1 min and kept at 50 °C for 30 min and then cooled to 4 °C to add 200 μL of 5% NH_3 in water. Then, the mixture was vortexed and centrifuged at 10000 rpm at 5 °C for 4 min to allow the layers to separate. The organic phase was analyzed by GC-MS (QP2010 GC/MS, Shimadzu, with an Rtx 5MS capillary column (20 m × 0.18 mm i.d. × 0.18 μm film). The temperature program was as follows: 50 °C initial hold for 1.5 min, increased to 150 °C at 50 °C/min, and then increased to 300 °C at 150 °C/min hold for 2 min. The split ratio was 10:1 with a helium flow under pressure of 103 kPa. Glycerol tris(trifluoroacetate) eluted at approximately 3.25 min. The mass spectrometer was operated in electron impact mode (70 eV), from 3.00 to 3.50 min (end time). Selective ion monitoring of m/z 267, 268, 269, 270, 271, and 272 was performed. The accuracy of this method was tested using a linear model fitted with the ratio of m/z 272 to 267 and the d_5-glycerol percentage (determination coefficient (r^2) = 0.99 covering the range from 0 to 12%).

Analysis of Cecal Microbiota. Microbiota profiling was assessed by barcode pyrosequencing as described previously,[30] with minor modifications. The method involves the amplification of two variable regions of the bacterial 16S RNA gene (V123 and V456) using a fixed primer at one end and a second primer at the other end carrying a unique tag of eight base pairs that allows assigning the sequence reads to a given sample. Bacterial DNA was extracted from the cecal digesta using a previously described phenol/chloroform bead-beating method.[31] Two PCR reactions were performed per sample, one for the V123 region and the second for the V456 region. For each set of primers, two 100 μL PCR reactions were prepared, containing 50 μL of Roche FastStart PCR Master (Roche, Basel, Switzerland), 40 pmol of each primer (ZyGEM, Hamilton, New Zealand), and a minimum of 2 ng of DNA template. PCR amplifications were performed using an Eppendorf Mastercycler ProS PCR System (Eppendorf, Hamburg, Germany). The PCR parameters were 94 °C for 5 min, 25 cycles of 94 °C for 30 s, and annealing at 49 °C for 30 s and at 72 °C for 30 s, followed by 72 °C for 7 min. After pooling of the two PCR products, 10 μL of the mix was visualized on agarose gel (1.2% in TBE buffer) stained with SYBR Safe (Invitrogen, Eugene, OR, USA). Then, PCR products were pooled in equimolar amounts and sent to Macrogen (Seoul, Korea) for sequencing using the GS-FLX Titanium System (Roche). Sequences were processed and binned as previously described.[30] Identification of 16S rRNA sequences (average length = 534 bp) was performed using the RDP Classifier with an 80% confidence threshold.

Data Analysis. The ability of animals to convert food intake into body weight was assessed by dividing the cumulative food intake for each animal by the corresponding animal weight (both intake and animal weight were measured three times per week during the 10 weeks).[32] The resulting data from week 5 onward (after the supplements were introduced into the diet) were compared to observe the effect of the treatments, using a generalized additive model, where the goodness of fit is presented in terms of R^2 and explained deviance.[33] This analysis identified what group of mice (if any) had significantly higher or lower ability to convert food intake into body weight compared to control group. Principal component analysis (PCA)[34,35] was applied to investigate the data obtained in direct infusion mass spectrometry. ANOVA was applied to test whether the treatment means were statistically different from the mean of the control group. When only one treatment was compared to control, Student's t test was used to compare the two means. The correlation between variables was estimated using Pearson's product moment correlation coefficient. Ellipse visualization[36] and correlation maps were used to compare the correlation between variables among different treatment groups. Data analysis was carried out in the software R.[37]

RESULTS AND DISCUSSION

Food Intake, Body Weight, and Metabolic Efficiency. In this study mice were supplemented with three different extracts, PL, SPL, or TPL). Food intake was not affected by the introduction of diet supplementation with milk polar lipids. During the first 5 weeks on a high-fat diet, animals consumed an average of 2.8 ± 0.2 g/day, whereas during the 5 weeks on supplementation of polar lipids, animals from control and test groups consumed an average of 2.6 ± 0.2 g/day of their corresponding diets. We did not observe any differences in final body weight (control, 20.08 ± 1.63 g; SPL, 20.24 ± 1.10 g; PL, 20.25 ± 1.60 g; TPL, 20.27 ± 1.04 g) or in average weight gain (control, 9.05 ± 5.51 g; SPL, 8.43 ± 3.13 g; PL, 12.03 ± 6.76 g; TPL, 9.39 ± 4.79 g) of the mice at the end of the experiment. The liver weights were not different ($P > 0.05$) among treatment groups (Supporting Information). Animals gained 37% in body weight in the first 5 weeks (i.e., pretreatment) and 10% in the last 5 weeks (see Tables S3 and S4 in the Supporting Information), whereas the reduction in food intake between the first and last 5 weeks was approximately 7% in

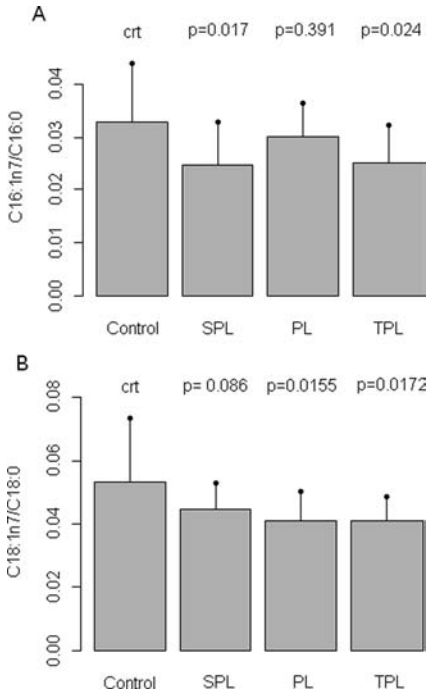

Figure 2. Ratio between the percentage of monosaturated fatty acids and corresponding saturated fatty acid detected in the liver. TPL, total polar lipid extract; PL, phospholipid-rich extract; SPL, sphingolipid-rich extract. Treatments were compared to control group using ANOVA, and resulting *P* values are shown above the bars.

Table 3. Fatty Acids Profile (Percent) in Brain Tissue of Animals from Control and TPL Groups[a]

	treatment group	
fatty acid	control	TPL
C16:0	13.84 (1.19)	14.08 (0.72)
C18:0	19.17 (0.97)	19.34 (1.10)
C18:1n9	11.25 (0.78)	11.25 (1.19)
C18:1n7	2.67 (0.11)	2.63 (0.19)
C18:2n6	1.15 (0.14)	1.13 (0.15)
C20:1n11	1.62 (0.34)	1.79 (1.03)
C20:1n9	0.49 (0.07)	0.49 (0.16)
C20:3n6	0.80 (0.15)	0.77 (0.17)
C20:4n6	12.28 (1.04)	12.39 (0.76)
C22:0	0.77 (0.16)	0.72 (0.15)
C22:4n6	4.67 (0.25)	4.58 (0.21)
C22:5n3	1.48 (0.34)	1.31 (0.38)
C22:6n3	24.18 (1.06)	24.37 (2.64)
C24:0	2.06 (0.42)	1.88 (0.32)
C24:1n9	3.56 (0.70)	3.25 (0.53)

[a]Values in parentheses represent standard deviation (n = 13). The difference in the percentage of individual fatty acid for the control and TPL groups was not statistically significant.

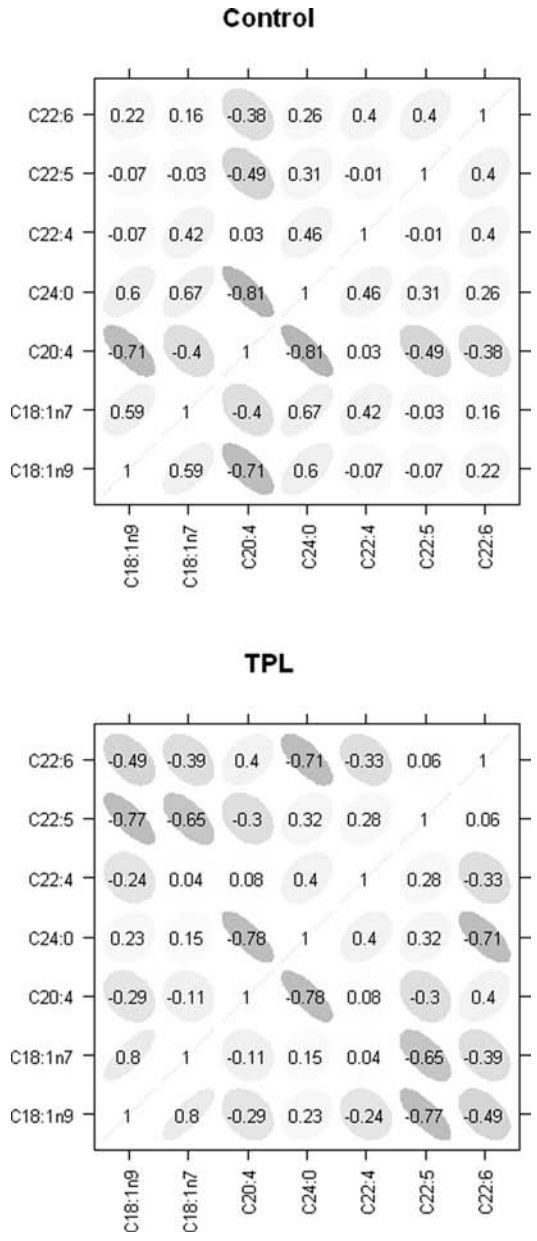

Figure 3. Pearson's product moment correlation coefficient between relevant fatty acids from brain samples. Each ellipse represents the correlation between a pair of fatty acids. The slope of the major axis of the ellipses indicates whether the correlation is positive (>0, bent to the right) or negative (<0, bent to the left). The size of the minor axis indicates the degree of correlation (high correlation = ∼1, narrow, no correlation = ∼0, circle). The shading is proportional to the absolute value of the correlation.

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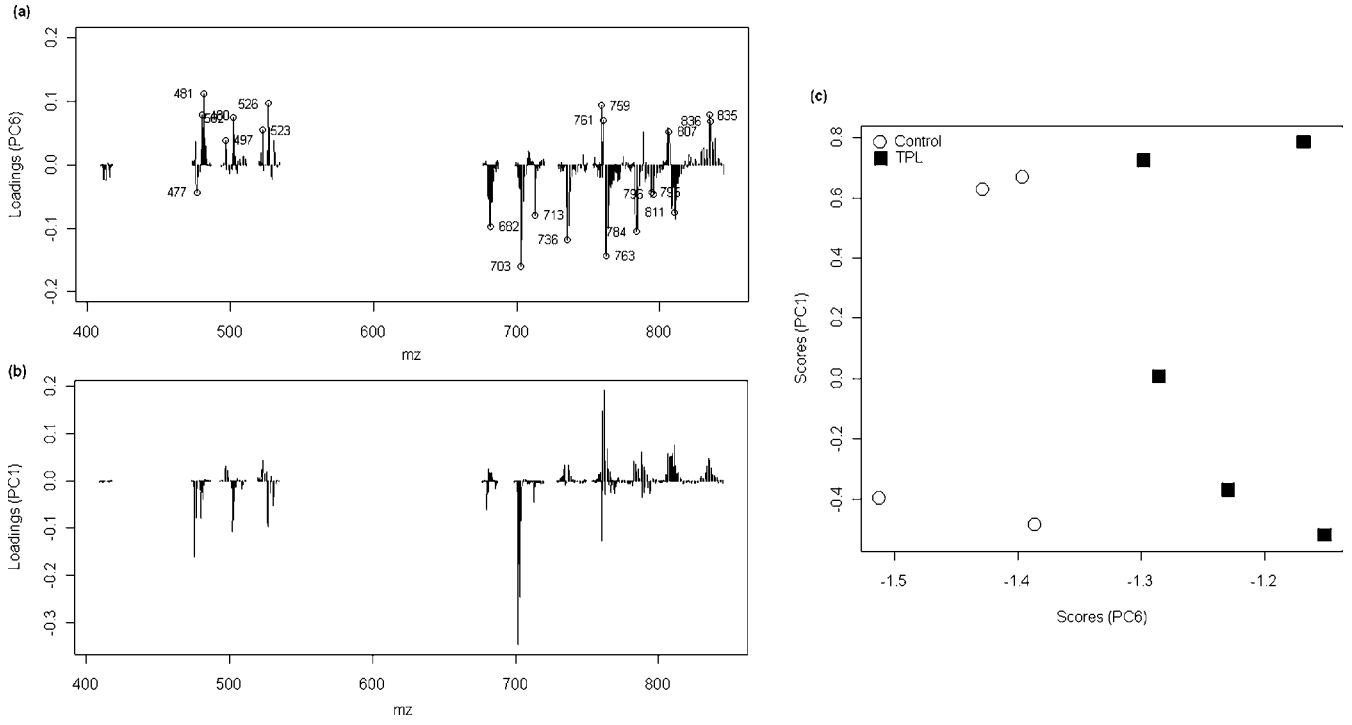

Figure 4. Principal component analysis of direct infusion data. Loadings of the sixth and first principal components are shown in panels a and b, respectively. Scores for these two principal components are shown in the scatter plot in panel c, where open circles represent samples from the control group and solid squares represent the TPL treatment group.

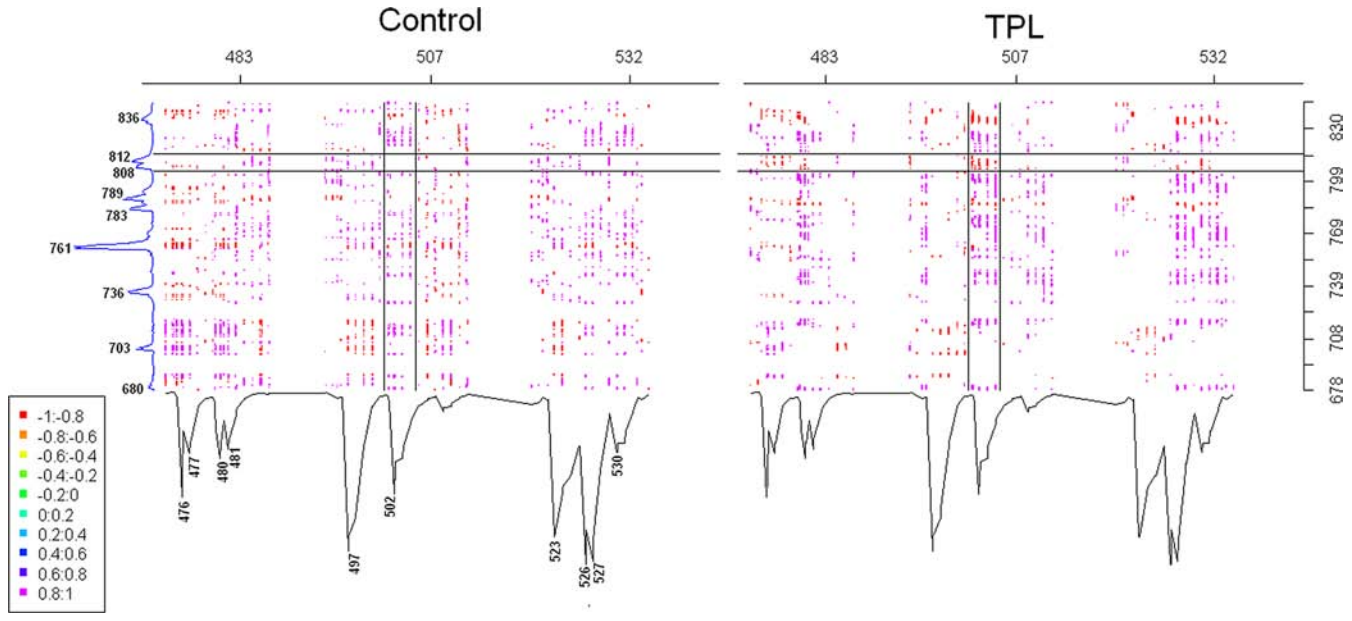

Figure 5. Pearson's product moment correlation coefficient between selected ions detected after direct infusion of lipid extract from brain tissue of the control and that of the TPL treatment group. Only significant correlations ($P < 0.05$) with absolute value >0.5 are shown. Vertical lines indicate m/z 501 and 505, and horizontal lines refer to m/z 808 and 812. The average spectrum corresponding to these two mass spectra ranges are shown at the bottom right of each plot. Colors represent the range of correlation values.

average. The ability of each animal to convert food intake into body weight was assessed by dividing the cumulative food intake for each animal by the corresponding animal weight.[38] TPL animals had lower ability to convert intake into weight gain when compared to control group ($P < 0.001$, $R^2 = 0.9$).

Lipid Metabolism in the Liver and Adipose Tissue. In the biosynthesis of lipids, hydrogen atoms from body water are incorporated in proportion to the amount of lipid synthesized. Thus, the fractional synthesis of lipids resulting from the de novo biosynthesis was evaluated in liver and adipose tissue using MIDA.[29] During administration of 4% ^2H_2O the level of ^2H in the urine remained at about 2.5% as shown in Figure S1 in the Supporting Information. Fractional synthesis of triglyceride in the liver (lipogenesis) tended to be reduced in the SPL group. This trend was not observed in the adipose tissue (Figure 1).

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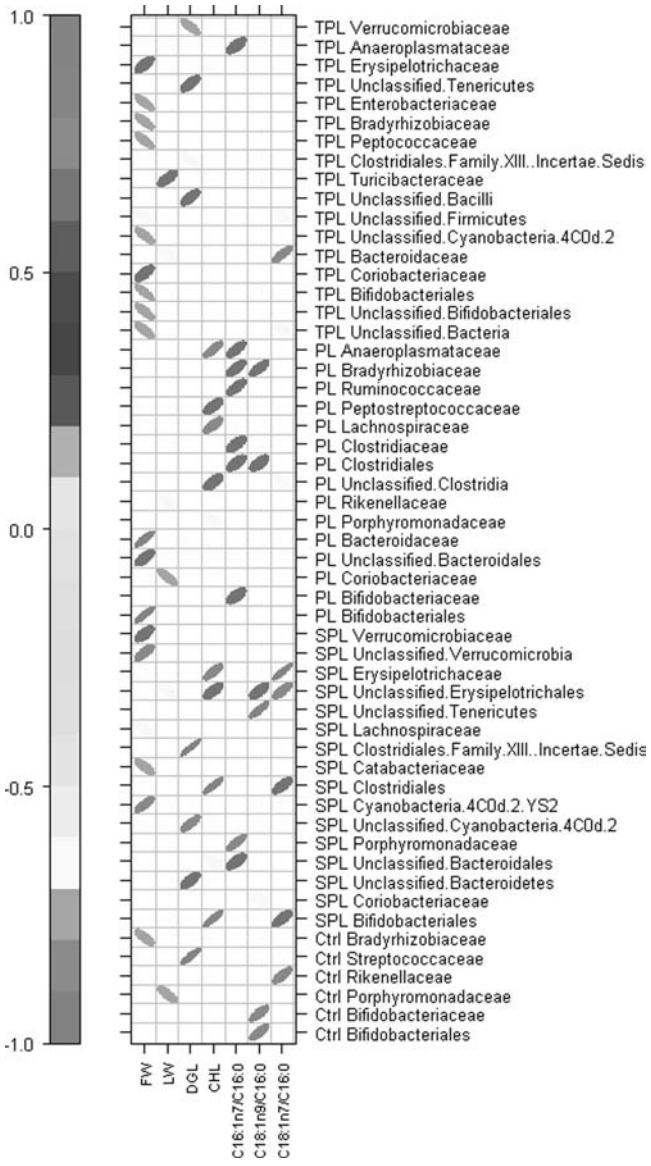

Figure 6. Pearson's product moment correlation coefficient between cecal microbiota (Family, V456) and phenotypes of the liver: final weight (FW), liver weight (LW), total triglycerides in the liver (DGL), free cholesterol (CHL), and ratio between monosaturated fatty acids and palmitate (C16:0) in different treatments group (TPL, total polar lipids; SPL, sphingolipids; PL, phospholipids) and control group (Ctrl). The slope of the major axis of the ellipses indicates whether the correlation is positive (>0, bent to the right) or negative (<0, bent to the left). The size of the minor axis indicates the degree of correlation (high correlation = ∼1, narrow). Only significant correlations (P < 0.05) with absolute value >0.5 are shown.

The palmitic acid (C16:0) in the liver can result from uptake from the diet or can be produced by de novo biosynthesis of fatty acids. A significant (P < 0.05) reduction in the number of ^{2}H atoms incorporated into the C16:0 was observed in the liver for the PL and TPL treatments when compared to control group (Figure 1). Thus, the reduction in the number of incorporated ^{2}H observed in this study indicates reduction in the de novo biosynthesis of fatty acids in the liver by the supplementation of milk polar lipids (PL and TPL). No effect on ^{2}H incorporation in the C16:0 was observed for the adipose tissue.

A significant (P < 0.05) reduction for the ratio of palmitoleic- (C16:1n7) to palmitic (C16:0) was observed in the liver of SPL and TPL groups when compared to control (Figure 2), but there was no effect on the C16:1n9/C16:0 ratio (data not shown). A significant (P < 0.05) reduction for the ratio of vacenic C18:1n7 (C18:1n7) to stearic (C18:0) was observed in the liver of PL and TPL groups when compared to control (Figure 2) but not for the ratio of oleic (C18:1n9) to C18:0 (data not shown). The ratio between C16:1n7 and C16:0 has been used as an indicator of the stearoyl-CoA desaturase (SCD1) activity that is responsible for the incorporation of one unsaturation in saturated fatty acids.[39−41] SCD1 catalyzes the synthesis of monounsaturated fatty acids, particularly C16:1n7 and C18:1n9,[42,43] whereas C18:1n7 is produced from elongation of C16:1n7. The reduction in the ratio C16:1n7/C16:0 indicates that reduction in the SCD1 activity was more accentuated for C16:1n7. Similarly, a reduction in the expression of SCD1 was observed by Wat et al.[9] investigating mice fed a high-fat diet supplemented with a dietary phospholipid-rich dairy milk extract and by Yunoki et al.[44] studying Zucker rats fed pure sphingomyelin of animal origin and glucosylceramide of plant origin. Shimizu et al.[45] observed antiobesity effects of phosphatidylinositol on diet-induced obesity in mice that also include the regulation of the expression of some genes in the liver involved in lipid metabolism, including the down-regulation of SCD1. Our study did not reveal a significant difference (P > 0.05) in free cholesterol and total fat in liver among treatments (see the Supporting Information). Our study differs from those of Shimizu et al.,[47] Wat et al.,[9] and Yunoki et al.,[46] as we started the supplementation after 5 weeks of high-fat diet stress. This may explain the lower effect of milk polar lipids observed because (i) animals were already under the high-fat diet stress and (ii) animals stayed for a shorter period under supplementation.

Our results suggest an additive effect of sphingolipids and phospholipids (TPL) on lipid metabolism of mice fed a high-fat diet, because smaller and different effects were observed for individual fractions (SPL and PL) (Figures 1 and 2). We did not observe any effect of the dairy polar lipid supplementation on the adipose tissue lipogenesis or adipose tissue fatty acid de novo biosynthesis.

Tandy et al.[46] observed that hydrogenated phosphatidylcholine supplementation reduced the level of hepatic lipid levels in mice fed a high-fat diet, including the down-regulation of sterol regulatory element binding protein 1-c (SPEBP-1c). This indicates that the effect observed for phospholipids is not associated with polyunsaturated fatty acid content. More recently, it has been observed that phospholipid ingestion ameliorates hypertriglycemia, hepatic steatosis, and glucose intolerance in rats fed a fructose diet in association with the reduction of a variety of lipids in the liver through the prevention of de novo lipogenesis, including down-regulation of SCD1.[43] Together, these studies suggest that sphingolipids and phospholipids are able to affect the lipid metabolism in the liver of mice fed a high-fat or high-sugar diet and that SCD1 might play a role in the biological activity of these polar lipids. Indeed, SCD1 has been shown to play an important role in the regulation of hepatic lipid metabolism, where its deficiency provides a protective effect against the development of hepatic insulin resistance in rats and mice on a high-fat diet.[42] The protective effect of SCD1 on hepatic steatosis has been attributed to a combined decrease in lipogenic rates and to the

activation of the β-oxidation pathway, although it is not clear how SCD1 affects and/or regulates lipogenic rates in liver.[47] Paton and Ntambi have proposed that deficiency of SCD1 leads to an inability to up-regulate de novo lipogenesis via SREBP-1c and storage of triglycerides.[45] Although alternative mechanisms for biological activity of sphingolipids and/or phospholipids under a high-fat diet have been proposed, such as a reduction in fat absorption during digestion,[7,48] the relationship between these polar lipids and SCD1 needs to be further addressed. The need for further studies is highlighted by the recent observation that phospholipids prevent fructose-induced alterations of the hepatic lipid profile by inhibiting de novo lipogenesis,[43] which cannot be explained by the hypothesis of reduction in fat absorption. We did not observe significant differences between treatment groups and control in the concentration of total triglycerides in the feces (data not shown).

Interestingly, a recent study shows that feeding ripened dairy products also down-regulates SPEBP-1c in obese and diabetic mice.[48] Although specific investigations are still required to identify the complex interaction between dairy product fermentation, dietary lipids, and lipid metabolism, this could involve the action of polar lipids.

Lipid Metabolism in the Brain. As the TPL treatment was the most active extract, samples from the brain of mice in this treatment were investigated further. The analysis of the percentage of individual fatty acids in brain tissue did not show differences between animals from the control and TPL groups (Table 3). However, Figure 3 shows the correlation coefficient between relevant fatty acids from brain samples, where the discrimination between these two treatments groups can be observed. In this case, the correlations between tetracosanoic acid (C24:0) and docosahexaenoic acid (DHA, C22:6) and between octadecenoic acid (C18:1) and docosapentaenoic acid (C22:5) showed the highest correlation values. The content of DHA in the brain correlated negatively with C24:0 in animals that were supplemented with TPL (-0.71, $P = 0.02$) but not for the control (0.26, $P = 0.44$). Arachidonic acid (AA, C20:4) was negatively correlated with C24:0 in both groups (TPL, -0.77, $P = 0.008$; control, -0.81, $P = 0.003$). The fatty acid C24:0 is mainly de novo biosynthesized in the brain, where it is found in relatively high amount as a component of sphingolipids.[49,50] Stranahan et al.[51] found that accumulation of C24:0 sphingomyelin in the hippocampus was elevated in rats on a high-fat and high-sugar diet when compared to control groups. We observed that the increase in de novo biosynthesis and/or accumulation of long-chain saturated fatty acid in the brain (measured by the increase in C24:0) is associated with a decrease in AA for both groups, but the decrease in DHA is observed only in the TPL group.

The lipid extract of a subset of randomly selected brain samples ($n = 4$ for control and $n = 5$ for TPL) was also investigated using direct infusion mass spectrometry. Samples were analyzed by ESI in positive ionization mode, and the identification of potential species was based on earlier studies with C57BL/6J mouse brain[52] and MS/MS published data.[53,54] Six major lipid classes were detected in positive ESI mode: phosphatidylcholines, phosphatidylserines, phosphatidylethanolamines, phosphatidylinositols, sphingolipids, and lysophospholipids (lyso-PLs). Principal component analysis was applied to data obtained in the MS full scan (Figure 4), where the discrimination between control and TPL groups can be observed in the scores of the sixth principal component (Figure 4c). The potential compounds responsible for these group separations are characterized mainly by two clusters of ions in the loadings for this principal component, formed by lyso-PLs and PLs (Figure 4a). The first principal component (Figure 4b) accounts for most of the explained variance (82%), and the sixth principal component accounts for a small portion of the variance (<1%), but its loadings are not randomly distributed (Figure 4a). Thus, further evaluation was performed to investigate this separation between groups. In this case, the correlation coefficients were estimated for each pair of variables (detected ions) in the m/z ranges of lyso-PLs and PLs (the estimated correlation coefficients were tested for significance). These results are presented as a correlation map in Figure 5, where each dot represents the correlation between one variable from the m/z ranges shown in the horizontal axis against a variable in the vertical axis. A highly significant negative correlation was observed between peaks in the ranges m/z 808−812 and 501−505 for the TPL group but not for the control group (Figure 5). The lipids lyso-PE-20:4 (m/z 502 is the expected ion in the ESI positive mode) and PE-20:4/22:6 (m/z 812 is the expected ion in the ESI positive mode) are two polar lipids that are found in the respective m/z ranges.[55] The negative correlation between these two peaks suggests the release of C22:6 from PE-20:4/22:6 to generate lyso-PE-20:4 (i.e., increase of lyso-PE-20:4 and decrease in C22:6) for the TPL group but not for the control group. These observations could help to explain our observations in the fatty acid analysis, where C22:6 decreases with increase of C24:0 for the TPL group compared to control, but the relationship between C20:4 and C24:0 remains constant in both groups; that is, C22:6 could have been released from PE-20:4/22:6 and metabolized.

It has been reported that a high-fat diet can lead to expression of inflammatory cytokines.[11] Pro-inflammatory cytokines are able to activate different phospholipase A_2 isoforms, leading to release of AA and DHA.[20,21] AA can be metabolized into potent pro-inflammatory mediators such as prostaglandins, leukotrienes, and hydroxyeicosatetraenoic acids, whereas the metabolites resolvins and protectins produced by DHA have an anti-inflammatory property.[20,21] In this context, this study presents an interesting observation suggesting the association between the increase in synthesis and/or accumulation of long-chain saturated fatty acids and the impact of dietary polar lipids on the metabolism of DHA, but not AA of mice fed a high-fat diet. This could be linked to a protective mechanism where DHA is metabolized for production of ant-inflammatory metabolites against the effects of a high-fat diet, although at this stage this is speculation and further work is required to investigate this hypothesis.

Analysis of Cecal Microbiota. Little effect of the polar lipid dietary supplementation on the composition of cecal microbiota was observed ($P > 0.05$). Figure 6 shows the correlation ($P < 0.05$) among some bacterial families and attributes of hepatic lipid metabolism and animal phenotypes (final body weight, liver weight); however, these correlations are not consistent across groups.

In conclusion, it is shown that PL and TPL were able to reduce de novo hepatic fatty acid biosynthesis ($P < 0.05$), whereas SPL and TPL were able to reduce the hepatic ratio between C16:1n7 and C16:0, which is an indirect indication of reduction of SCD1 activity ($P < 0.05$). These results indicate that phospholipids and sphingolipids may act in different steps of the hepatic lipogenesis. No effect was observed in the adipose tissue, and little effect was observed on the gut microbiota composition. In the brain, TPL supplementation

was able to affect the metabolism of DHA, but not AA. This may be linked to a protective mechanism whereby DHA is metabolized for production of anti-inflammatory metabolites against the effects of a high-fat diet. Therefore, bovine milk total polar lipids have the potential to be used as a functional ingredient to ameliorate the impact of a diet rich in fat, where further separation or enrichment of individual polar lipids classes is not needed. Nevertheless, the effects of these polar lipids on brain and other tissues under the stress of a high-fat diet need to be further explored.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures S1–S3 and Tables S1–S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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