HOKKAIDO UNIVERSITY

Title	The dietary effect of milk sphingomyelin on the lipid metabolism of obese/diabetic KK-A(y) mice and wild-type C57BL/6J mice
Author(s)	Yamauchi, Ippei; Uemura, Mariko; Hosokawa, Masashi; Iwashima-Suzuki, Ai; Shiota, Makoto; Miyashita, Kazuo
Citation	Food & function, 7(9), 3854-3867 https://doi.org/10.1039/c6fo00274a
Issue Date	2016-07-26
Doc URL	http://hdl.handle.net/2115/66766
Type	article (author version)
File Information	Miyashita FF.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

The dietary effect of milk sphingomyelin on the lipid metabolism of obese/diabetes KK-A^y mice and wild-type C57BL/6J mice

Ippei Yamauchi[a], Mariko Uemura[a], Masashi Hosokawa[a], Ai Iwashima-Suzuki[b], Makoto Shiota[b] and *Kazuo Miyashita[a]

[a]Laboratory of Bio-functional Material Chemistry, Division of Marine Bioscience, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

[b]Megmilk Snow Brand Co. Ltd., 1-1-2 Minamidai, Kawagoe, Saitama 350-1165, Japan

Running title: The effect of milk sphingomyelin

*Author for correspondence: Kazuo Miyashita, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan.
E-mail: kmiya@fish.hokudai.ac.jp

Purified milk sphingomyelin (SM) was obtained from lipid concentrated butter serum (LC-BS) by successive separations involving solvent fractionation, selective saponification, and silicic acid column chromatography. The SM obtained was given to obese/diabetic KK-A^y mice and wild-type C57BL/6J mice. SM supplementation significantly increased fecal lipids paralleled with a decrease in non-HDL cholesterol levels in the serum and neutral lipids and in cholesterol levels in the livers of KK-A^y mice. The reduction of liver lipid levels also resulted in a decrease in the total fatty acid content of the KK-A^y mice livers, while n-3 fatty acids derived from the conversion of α-linolenic acid (18:3n-3) increased due to SM supplementation. In contrast to the KK-A^y mice, little change in the serum and liver lipids was observed in wild-type C57BL/6J mice. The present study suggests that SM may be effective only in subjects with metabolic disorders.

Introduction

The milk fat globule membrane (MFGM) is a biological membrane that surrounds milk fat droplets. It prevents the globules from coalescence, stabilizes them in the milk serum, and protects them from enzymatic attack by lipases.[1] MFGM is a mixture of bioactive proteins and polar lipids. Mechanical treatments induce the release of MFGM from fat globules into the corresponding serum phase (i.e., buttermilk and butter serum). Butter serum is produced from butter to anhydrous milk fat production and is rich in proteins and phospho- and sphingolipids from MFGM. The major phospholipids (PL) are

phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine, and phosphatidylinositol, while the major sphingolipids (SL) are glucosylceramide, lactosylceramide, and sphingomyelin (SM).

Milk nutrients have attracted attention as functional foods and nutraceuticals with potentially important cardioprotective properties. Recent studies showed that increased consumption of milk and dairy products is associated with a reduced incidence of obesity, insulin resistance, dyslipidemia, and type 2 diabetes, which are cardiovascular risk factors.[2] Among the milk component, polar lipids rich in butter serum have been considered active components in the improvement of lipid metabolism. The hypolipidemic and/or hypocholesterolemic activity of milk PL and SL have been found in animal models.[3-5] Wat *et al.*[5] reported a significant decrease in the liver weight, total liver lipid, liver triacylglycerol (TAG), and total cholesterol and serum lipids of mice fed a high-fat diet with a PL-rich dairy milk extract. Milk PL supplementation could also significantly decrease the total liver cholesterol and TAG levels of mice (C57BL/6) fed a high-fat diet.[4] On the other hand, we have found a significant decrease in the plasma cholesterol, hepatic total cholesterol and TAG levels of obese/diabetic mice (KK-A^y) by the supplementation of lipid concentrated butter serum (LC-BS). Furthermore, when ceramide, SM, PE, and PC rich fractions from LC-BS was given to the KK-Ay mice, significant decrease in plasma cholesterol and hepatic lipid levels was found in the animals fed ceramide fraction. [5] The decrease was also found in the mice fed SM fraction. On the other hand, there was little effect of PE and PC fractions on the lipid levels. This result suggested that the effect of LC-BS is mainly due to SL, the main components of LC-BS.[5] However, purity of ceramide and SM fractions used in the study was 70.7 and 49.9% respectively. A human study has also demonstrated the possible effect of the intake of milk polar lipids on cholesterol absorption from the intestine and/or hepatic metabolism.[6]

Although several studies have demonstrated the hypolipidemic and hypocholesterolemic activity of milk polar lipids, there has been no study on the effect of purified lipid classes from milk polar lipids. Moreover, the mechanism for the activity of the milk polar lipid on lipid metabolism has not been made clear. Thus, in the present study, we separated SM from LC-BS and measured its dietary effect on serum, liver, and fecal lipid contents by using obese/diabetic KK-A^y mice and wild-type C57BL/6J mice.

Materials and Methods

SM preparation

Butter serum was treated with acidic pH to remove milk protein. Subsequently, the products were ultrafiltrated in an industrial scale to obtain commercial LC-BS by the Snow Brand Milk Products Co., Saitama, Japan.[7] The LC-BS prepared had still more than 40% of non-lipid components including protein protein (>20%), carobohydrate (>10%), and ash (>5%). Thus, the LC-BS was extracted with 10 volumes (v/w) of chloroform/methanol (2:1, v/v) and allowed to stand overnight. The solution was filtrated, and the filtrates were concentrated under a vacuum using a rotary evaporator to obtain butter serum lipids. The butter serum lipids contained SL, PL, and neutral lipids such as triacylglycerols and sterols.[5] The first separation of butter serum lipids was carried out on the basis of the insolubility of polar lipids such as SL and PL in acetone and diethyl ether. Then, ten volumes of acetone (v/w) were added to the butter serum lipids and allowed to stand overnight. The precipitate was recovered by centrifugation at 1260 g for 5 min and dissolved again in 10 volumes (v/w) of diethyl ether. Crude milk polar lipids were precipitated by leaving the solution overnight followed by centrifugation at 1680 g for 10 min. The next step was the removal of glycerol-*o*-esters, such as PL. These glycerol-*o*-acyl esters could be saponified in the weak alkaline condition used in the present study, while N-acyl esters such as SL were resistant to the saponification. Then, the lipids were saponified with 200 volumes of 0.2 N NaOH in methanol at 37°C for 20 min to remove these glycerol-*o*-esters. After neutralization with 2.6 N HCl in methanol, the unsaponifiable fraction was dissolved in chloroform/methanol/water (10:5:3, v/v/v). The solution was placed into a separatory funnel and was shaken vigorously. After allowing the funnel to stand overnight, the lower layer was evaporated under reduced pressure in a rotary evaporator. The unsaponifiable matters (*ca*. 10 g), mainly SL, were passed through a column packed with a chloroform/methanol/water (65:25:4, v/v/v) slurry mixture of Silica gel BW-80S (Fuji Sylysia Chem. Ltd., Kasugai, Aichi, Japan) (700 g) by eluting the same solvent. The fraction eluted with the solution (500 mL) was fractionated and the effluent was analyzed using thin-layer chromatography (TLC). The TLC was performed on a 0.25 mm silica gel plate (Silica gel 60G; Merck) developed with chloroform/methanol/water (65:25:4, v/v/v). The lipid spot was detected with the Dittmer reagent.[8] Identification of the spot was performed using standard milk SM. The SM fractions were combined and concentrated. Standard SM from milk was obtained from Nagara Science Co., Ltd., Oritate, Gifu, Japan.

Analysis of SM

The purity of the SM fraction obtained as described above was analyzed by TLC and

high-performance liquid chromatography (HPLC). The lipid fraction was dissolved in chloroform-methanol-water (65:25:4, v/v/v) solution and analyzed by TLC as described above. The spot was visualized by spraying with the Dittmer reagent, followed by charring all spots at 150°C.

The purity of SM was also analyzed using HPLC. HPLC was performed with a Shimadzu HPLC system (Shimadzu Seisakusho, Kyoto, Japan) equipped with a pump (Shimadzu LC-20AD) and an evaporated light scattering detector (Hitachi ELSD-LT II). The analysis was performed on a silica column (Mighttysil Si 60, 250 x 4.6 mm i.d; Kanto Chemical Co. Ltd., Tokyo, Japan). The mobile phase consisted of dichloromethane (A) and methanol/water (95:5, v/v) (B). A gradient elution procedure was programmed as follows: 0-5 min, 99:1 (A:B, v/v); 20-25 min, 80:20 (A:B, v/v); 35-40 min, 10:90 (A:B, v/v); and 40-45 min, 1:99 (A:B, v/v). A linearly programmed gradient went from 99:1 (A:B, v/v) to 80:20 (A:B, v/v) at 5-20 min and from 80:20 (A:B, v/v) to 10:90 (A:B, v/v) at 25-35 min, respectively. The flow rate was kept at 1.0 mL/min and the column temperature was maintained at 40°C. Each sample (*ca.* 10 mg) was dissolved in dichloromethane/methanol (1:1, v/v) and 1 μl was injected onto HPLC. The drift tube temperature was 50°C and the nebulizer gas (N_2) pressure was 350 kPa. Milk SM was used as the standard.

Animals and diets

Obese/diabetic KK-A^y mice (male, four weeks old) and wild-type C57BL/6J mice (male, four weeks old) were obtained from Japan CREA Co., Tokyo, Japan. The KK-A^y mice were housed individually, while C57BL/6J mice of the same experimental group (n=6) were housed in one cage. They had free access to food and tap water. Room temperature and humidity were controlled at 23 ± 1˚C and 40-60% with a 12 h light/12 h dark cycle. After acclimation for a week, including being fed a normal rodent diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan), the mice were randomly divided into groups of seven (KK-A^y mice) or six (C57BL/6J mice) and were then fed experimental diets for four weeks. The body weight, diet and water intake of each mouse was recorded daily. The composition of the diets is shown in Table 1. Several studies[3-5] examined the effect of milk polar lipids mainly rich in PL. These lipids also contained significant amounts of SM and the SM level was from 0.25 - 0.55 wt% of total diet. The lipid content of SM in the dietary lipids has been up to 12% in these studies. To make clear the dietary effect of SM, 1.0% of SM (14% of the dietary lipids) was adapted in the present study.

Ethics

The research project was approved by the Ethical Committee at Hokkaido University and all procedures for the use and care of animals for this research were carried out under the approval by the Ethical Committee of Experimental Animal Care at Hokkaido University.

Fatty acid composition of dietary lipids

Dietary lipids, lard, soybean oil, linseed oil, and fish oil, were obtained from Showa Chemical Industry Co. Ltd., Tokyo, Wako Pure Chemical Ind., Osaka, Summit oil Mill Co. Ltd., Chiba, Junsei Chemical Co. Inc., Tokyo, and Maruha Nichiro Co., Tsukuba, Japan, respectively. After the dietary lipids were mixed with other dietary ingredients (Table 1), the lipids were extracted from the diets with chloroform/methanol (2:1, v/v) as described previously by Folch *et al*.[9] The fatty acid composition of the extracted lipids was determined by gas chromatography (GC) after the conversion of fatty acyl groups in the lipids to their corresponding methyl esters. Two milliliters of 5% HCl-methanol were added to a sample lipid (20-50 mg) followed by incubation at 100°C for 3 h. The HCl-methanol solution was prepared by the dilution of 10% HCl-methanol solution. After cooling the solution, 2 mL of water were added and vortexed, followed by the addition of 2 mL of *n*-hexane. The upper hexane layer containing the methyl esters was recovered and the residual lower layer was further extracted with 2 mL of *n*-hexane. The hexane extracts were combined and washed with water to achieve neutrality. After concentrating the hexane solution under a vacuum, the methyl ester obtained was purified on a silica gel column (silica gel 60; Merck) in an elution with *n*-hexane and a mixture of *n*-hexane-diethyl ether (95:5, v/v). Purified methyl esters were subjected to GC analysis. GC was performed on a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column [Omegawax 320 (30 m x 0.32 mm i.d.); Supelco, Bellefonte, PA]. The injection port and flame ionization detector were set at 250 and 260°C, respectively, and the column temperature was held at 200°C. The carrier gas was helium at a flow rate of 50 kPa. Fatty acid content in the lipid samples was expressed as a weighted percentage of the total fatty acids.

Sample collection

Blood samples were taken from caudal vein of the mice without fasting at 0, 7, 14, 21, and 28 days after feeding. Blood glucose was measured using a blood glucose monitor, the Glutest Neo Sensor (Sanwa Kagaku Kenkyusyo Co. Ltd., Aichi, Japan). This sensor is an amperometric sensor with flavin adenine dinucleotide (FAD)-dependent glucose

dehydrogenase and $Fe(CN)_6^{3-}$. After feeding with the experimental diets for four weeks, the mice were sacrificed under diethyl ether anesthesia. Blood samples were taken from the caudal vena cava of the mice and each tissue was immediately excised and weighed. The livers were immediately stored in RNA later™ (Sigma Chemical Co., St. Louis, MO) for quantitative real time PCR analysis.

Blood lipid analysis

The blood serum analysis of the KK-A^y mice was performed by the Analytical Center of Hakodate Medical Association (Hakodate, Japan). The analysis included the measurement of the following parameters: total cholesterol, neutral lipids (NL), PL, Non-HDL cholesterol, LDL cholesterol, and HDL cholesterol. Blood serum from C57BL/6J mice was extracted with chloroform/methanol (2:1, v/v) according to the method by Folch et al. (1957). The total lipids (TL) extracted were weighed and the serum TAG and cholesterol content were enzymatically measured using commercial kits (Cholesterol E-test and Triglyceride E-test, Wako Pure Chemical Industries Ltd., Osaka, Japan).

Some period of fasting is required before glucose tolerance test and needed to obtain stable baseline measurements of blood lipid parameters; however, several recent studies have demonstrated the adverse effect of fasting in rodents.[10,11] During the fasting, especially overnight fasting, they consume much calories and prolonged fasting inhibits insulin-stimulated glucose uptake in humans, but increases the insulin sensitivity in mice. In the present study, we used non-fasting mice for the analysis of blood glucose and serum lipid parameters.

Liver Lipid Analysis

TL was extracted from the liver (*ca.* 200 mg) with chloroform/methanol (2:1, v/v).[9] The TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge (Waters Japan, Tokyo, Japan) by elution with chloroform (50 mL) and methanol (50 mL). The NL and PL fractions were eluted with chloroform and methanol, respectively. Both lipid contents (mg/g liver) in the liver were calculated from the TL level per liver weight. The TAG and cholesterol content in the TL were enzymatically measured using commercial kits as described above.

The fatty acid methyl esters from the liver TL were prepared using the method of Prevot and Mordret.[12] Briefly, 1 mL of *n*-hexane and 0.2 mL of 2 N NaOH in methanol were added to an aliquot of total lipid (*ca*. 10 mg), vortexed and incubated at 50°C for 30 min. After the incubation, 0.2 mL of 2 N HCl in methanol solution was added to the

solution and vortexed. The mixture was separated by centrifugation at 1000 g for 5 min. The upper hexane layer containing fatty acid methyl esters was recovered and subjected to GC. The GC was performed as described above. Each fatty acid level of the liver tissue (1 g) was calculated by comparing the peak ratio to that of the internal standard (17:0) and the total lipid content.

Faces analysis

Feces excreted during whole day (24 hr) were collected from a metabolic cage one time (27 day after feeding) for KK-A^y mice and two times (15 or 29 day after feeding) or three times (1, 14, or 27 day after feeding) for C57BL/6J mice. The KK-A^y mice were housed individually; therefore, feces of three days for each animal were individually analyzed. On other hand, C57BL/6J mice of the same experimental group (n=6) were housed in one cage. Thus, feces of 6 animals were analyzed all together. KK-A^y mice were Collection was done and freeze-dried. After freeze-drying and recording the weight, the samples were further dried in a vacuum desiccator and subsequently crushed to pieces in a coffee mill. The lipids were extracted from the dried powder with 10 volumes (v/w) of chloroform/methanol (2:1, v/v) and allowed to stand overnight. The solution was filtrated and the filtrates were concentrated under a vacuum using a rotary evaporator. After weighing the feces lipids, a part of the lipids (*ca*. 1 mg) was subjected to the measurement of total cholesterol and total bile acid using the Cholesterol E-Test (Wako Pure Chemical Industries Ltd.) and the Bile Acid Test Wako (Wako Pure Chemical Industries Ltd.), respectively.

Quantitative Real-Time PCR

Total RNA was extracted from the livers of mice using RNeasy Lipid Tissue Mini Kits (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real-time PCR analyses of individual cDNA were performed with ABI Prism 7500 (Applied Biosystems Japan Ltd., Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan). The mRNA analyses were performed on genes associated with lipid metabolism, which included sterol regulatory element-binding protein 2 (SREBP-2), hydroxymethylglutaryl-CoA reductase (HMG-CoA), cytochrome P450 7A1 (Cyp7a1), Carnitine Palmitoyltransferase 1A (CPT1a), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD1), elongase-2 (Elov2), elongase-5 (Elov5), Δ^5-desaturase (Fads1), and Δ^6-desaturase (Fads2). The gene-specific primers were Mm01306292_m1

(SREBP-2), Mm01282499_m1 (HMG-CoA), Mm00484152 (Cyp7a1), Mm00550438_m1 (CPT1a), Mm00662319_m1 (FAS), Mm00772290_m1 (SCD1), Mm00517086_m1 (Elov2 mRNA), Mm00506717_m1 (Elov5 mRNA), Mm00507605_m1 (Fads1 mRNA), Mm00517221_m1 (Fads2 mRNA), and Mm99999915_g1 (GAPDH mRNA; internal control), respectively.

Statistical analysis

Data are presented as the means ± standard error of the mean (SEM) (n=7 or 6). The data were analyzed by a two-way Analysis of variance (ANOVA) using SM and dietary lipids as two variable factors. When no interaction was present between both factors, different groups were compared by Tukey's post hoc analysis. If an interaction was present, one-way ANOVA and t-test were performed between two groups fed the same dietary lipid with or without SM. Differences with $P < 0.05$ were considered significant.

Results

SM separation

Commercial LC-BS contained 51.9 ± 0.69 wt% lipids per dry matter (average ± standard deviation of the mean, n=3). Crude milk SL were concentrated from the LC-BS on the basis of the different distribution of milk lipid class to organic solvents, and then were further separated by selective saponification to remove glycerol-*o*-acyl esters, such as phospholipids. The crude SL (5.51 ± 0.49 g) was recovered from 100 g of LC-BS. Purified SM (1.89 ± 0.53 g) was obtained from the crude SL by silicic acid column chromatography. The SM gave only a single spot and a single peak corresponding to the standard milk SM on TLC and HPLC, respectively. Fatty acid analysis by GC showed that the major fatty acids of SM were long chain saturated fatty acids such as 22:0, 23:0, and 24:0. The GC analysis also showed the major fatty acids of lard (18:1n-9, 16:0, and 18:0), soybean oil (18:2n-6 and 18:1n-9), linseed oil (18:3n-3, 18:1n-9, and 18:2n-6), and fish oil (22:6n-3 and 20:5n-3). The fatty acid profile of the lipids extracted from each diet is shown in Table 2. The composition was reflected by the dietary lipids with or without SM (Table 1).

Body weights, tissue weights, blood glucose levels, serum and hepatic lipid parameters.

There were significant differences in several parameters, namely, water intake and liver weight for KK-A^y mice, and food intake, water intake, liver weight, kidney weight, and

small intestine weight for C57BL/6J mice (Table 3). On the other hand, no significant difference was found in body weight, total white adipose tissue (WAT) weight, and blood glucose level in both animal models. There was also no significant difference in the blood glucose levels at 0, 7, 14, 21, and 28 days after feeding.

On the other hand, serum total cholesterol, PL, non-HDL cholesterol, and LDL cholesterol levels were affected by the dietary lipids in KK-A^y mice, although little effect was found in the levels of NL and HDL cholesterol (Fig. 1). SM supplementation decreased non-HDL cholesterol levels in soybean oil- and linseed oil-fed mice (Fig. 1 D). The same tendency was also observed in total cholesterol (Fig. 1 A) and phospholipids (Fig. 1 C). When the comparison was done between the two groups fed the same dietary lipids with or without SM, the significant decrease in the LDL cholesterol levels was found by SM supplementation in soybean oil- and linseed oil-fed mice (Fig. 1 E). A similar dietary effect was found in the liver lipid content (Figure 2). The reducing effect of SM supplementation was found on the TL (Fig. 2 A), NL (Fig. 2 B), TAG (Fig. 2 D), and cholesterol (Fig. 2 E) levels in the liver of KK-A^y mice, though there were no significant differences. The soybean and linseed oil fed groups had significantly lower levels of TL and NL than the lard group. Overall, Fig. 1 and 2 indicate the combined effect of SM supplementation with dietary fat containing polyunsaturated fatty acids (PUFA), such as linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) (Table 2) on the reduction of serum and liver lipids. In contrast to the KK-A^y mice, no decrease in serum and liver lipids was observed in the C57BL/6J mice (Fig. 3 and 4).

Fecal lipids.

The fecal concentrations of TL, cholesterol, and PL were also affected by dietary lipids (Fig. 5 and 6). In the KK-A^y mice, the fecal TL content was the lowest in the linseed oil-fed group; however, the level (88.7+41.2 mg/g feces) significantly increased to 221.7±27.6 mg/g feces following SM supplementation (Fig. 5 A). The excretion of total lipids in the feces was promoted by SM supplementation in the lard- and soybean-fed groups. Fecal cholesterol levels were also increased by SM supplementation and significant difference between with or without SM diets were found in the lard- and linseed oil-fed groups (Fig. 5 B). Bile acid was also significantly increased by SM supplementation in lard-fed group, while there was no significant effect of SM supplementation in other two groups (Fig. 5 C). The increase in fecal TL was also found in C57BL/6J mice (Fig. 6). Although statistical analyses could not be performed because the experimental groups with C57BL/6J mice were housed in the same cage,

the result in Fig. 6 strongly suggests the greater excretion of TL in the feces following SM supplementation in C57BL/6J mice. To confirm the promotion of TL secretion into the feces in C57BL/6J mice by SM supplementation, separate animal experiments have been performed using dietary lipids containing 230 g/kg lard and 70 g/kg soybean oil or 60 g/kg soybean oil + 10 g/kg SM. When both diets (Table 1) were given to wild-type C57BL/6J mice (male, four weeks old, n=7) for 29 days, a significant increase in the TL in the feces following SM supplementation was observed 15 and 29 days after feeding (Fig. 7).

Fatty acid levels of liver lipids and gene expression

Lard diets contained higher levels of saturated and monounsaturated fatty acids, such as 16:0 and 18:1n-9, than the other dietary groups (Table 2). Thus, high levels of 16:0 and 18:1n-9 were found in the liver lipids of lard-fed KK-A^y mice (Table 4). The characteristic fatty acid compositions of the soybean oil and linseed oil groups were high levels of LA and ALA, respectively (Table 2). Both PUFA were also found at relatively high concentrations in the liver lipids of soybean oil- and linseed oil-fed KK-A^y mice, respectively (Table 4). On the other hand, SM supplementation significantly reduced the total fatty acid content (mg/1 g liver) of the lard-fed mice. The same tendency was found in the total fatty acid contents of the soybean oil- and linseed oil-fed mice. The decrease in the total fatty acids, presented in Table 4, was consistent with the result in Fig. 2 showing the reducing effect of the SM supplementation on liver TL (Fig. 2 A) and NL (Fig. 2 B). Table 4 also shows the decrease in saturated and monounsaturated fatty acids, LA, and ALA by SM supplementation; however, arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA), docosapentaenoic acid (22:5n-3, DPA), and docosahexaenoic acid (22:6n-3, DHA) increased in the SM supplemented soybean oil- and linseed oil-fed groups.

To determine the effect of dietary lipids on liver lipid metabolism in KK-A^y mice, the related gene expressions were analyzed by real-time PCR. The analysis showed no significant effect of dietary lipids on the gene expression related to cholesterol metabolism (SREBP-2, HMG-CoA, and Cyp7a1) (Fig. 8) and on the expression of FAS and CPT1a. On the other hand, a difference was found in SCD1 gene expression, with a conversion from 16:0 and 18:0 to 16:1n-7 and 18:1n-9. Table 4 presents the significantly higher levels of 18:1n-9/18:0 in the lard-fed group compared to the soybean and linseed oil-fed groups. On the other hand, a significant decrease in 16:1n-7/16:0 was observed due to SM supplementation in the soybean oil-fed group. The decreasing trend in 18:1n-9/18:0 was also observed due to SM supplementation in

soybean oil- and linseed oil-fed groups. The change in the ratio of monoenoic fatty acid/saturated fatty acid was consistent with the dietary up- and down-regulation of SCD1 mRNA presented in Fig. 9. LA and ALA are converted to ARA and DHA, respectively, through a series of desaturation and chain elongation processes including Elov2, Elov5, Fads1, and Fads2. The different diet feeding resulted in the significant changes in the expression of Elov2, Elov5, and Fads2 (Fig. 9), while no significant difference was found in the expression of Fads1.

On the other hand, no decrease in the liver total fatty acids was observed in C57BL/6J mice following SM supplementation (Table 5). The fatty acid composition of the liver lipids was well reflected by the dietary lipids to show the high level of ALA and DHA in the linseed oil- and fish oil-fed group, respectively; however, no specific effect of SM supplementation was found on the fatty acid composition including 18:1n-9/18:0 and 16:1n-7/16:0 ratio. In addition, there was no significant difference in gene expression related to lipid metabolism with or without SM supplementation.

Discussion

SM is an essential biological component with important roles, such as cell membrane formation, lipid microdomains functionality, and signal transduction.[14-15] On the other hand, SM is a dietary component with an average consumption per capita in the Western diet of ~200-400 mg/day.[16,17] Studies have examined the effects of dietary SM and have found reductions of the liver and plasma lipid levels.[18] In the present study, we also found that dietary SM could reduce the liver and plasma lipid levels of obese/diabetic KK-A^y mice (Fig. 1, 2, and Table 4). This effect was mainly dependent on the increase in fecal TL and cholesterol observed in the mice fed SM (Fig. 5). Inhibition of the intestinal lipid absorption by SM has also been reported as the probable mechanism for the lowering effect of SM on the liver and/or plasma lipid levels.[19-22] This effect of SM is due to its physical property of being relatively resistant to solubilization into bile salt micelles. The low solubility of SM induces its incomplete hydrolysis in the upper segment of the intestine, where much of lipid hydrolysis occurs.[23] The slow and incomplete hydrolysis of SM may allow for interactions between intact SM and other lipids in the luminal environment, lowering the rates of hydrolysis, micellar solubilization and the transfer of lipids from mixed micelles to the enterocyte.[21]

Although the inhibition of the intestinal lipid absorption by dietary SM has been made clear using physico-chemical model,[24] cellular model,[25,26] ^{3}H-dihydrosphingosine-labeled SM,[17] and lymph cannulation method,[21]

research of the effect of dietary SM in animal models has been limited. Duivenvoorden *et al.*[18] reported the lowering effect of SM on plasma lipid levels of hyperlipidemic *APOE*3*Leiden mice fed a Western-type diet. The same effect has been reported in obese Zucker rats.[22] The present study also confirmed the reduction of serum and liver lipid levels in obese/diabetic mice through the promotion of intestinal lipid secretion by SM. Dietary SM also promoted fecal lipids in wild-type C57BL/6J mice (Fig. 6 and 7); however, no decrease in serum and liver lipids was observed in the wild-type mice (Fig. 3 and 4). This may suggest the resistance of normal conditions to changes in the lipid profiles of biological systems. Our present finding on the different effect of SM on obese/diabetic and wild-type model mice suggests the possibility of effectiveness of SM on human subjects with metabolic abnormalities. However, cholesterol metabolism of mice is different from that of human. A human study reported no significant changes in the plasma lipid profile after the consumption of SM.[23,27,28] Ramprasath *et al.*[23] demonstrated some limitations of human studies: e.g., sample size, the SM containing diet formulation, and the dose level of SM. In addition, these human studies have been conducted with only healthy subjects; therefore, more studies to determine whether SM affects cholesterol absorption and plasma lipids in hyperlipidemic subjects are needed.

Recently, much attention has been paid to the health beneficial effects of milk SM.[29-31] When milk SM was given to C57BL/6J mice fed high fat diet, significantly reduction was found in body weight, serum cholesterol and hepatic triglycerides levels.[29] On the contrary, the same level of egg SM supplementation increased the serum cholesterol, triglycerides, phospholipids, and hepatic triglycerides.[29] Lecomte *et al.*[31] reported that the supplementation soybean polar lipids to C57BL/6J mice fed high fat diet significantly increased the hepatic lipid levels, while there was little effect of milk polar lipids on the hepatic lipid levels. It is apparent that supplementation of SM including egg and soybean SM could inhibit lipid absorption in animal models.[19,21,24-26,32] To compensate for the reduction of absorbed lipids, hepatic de novo lipogenesis would be up-regulated. Norris *et al.*[29] found that milk SM feeding significantly increased hepatic HMG-CoA and SREBP2 gene expressions of C57BL/6J mice fed high fat diet. On the other hand, milk SM supplementation significantly decreased serum total cholesterol and hepatic TAG, although the reverse effect was found in egg SM supplementation.[21,29] Moreover, egg SM feeding significantly increased SCD1 gene expressions, while no increase in SCD1 was found in the mice fed milk SM.[29] In the present study, milk SM supplementation decreased SCD1 of KK-A^y mice (Fig. 9A), while the increase in HMG-CoA and SREBP2 gene expressions was observed (Fig. 8 A and B).

ALA is an essential fatty acid that must be consumed through diet. There have been many epidemiological and clinical studies on the cardiovascular-protective effects of ALA.[33] LA is a precursor of EPA and DHA. Both n-3 EPA and DHA have been regarded as active forms of ALA in biological systems. EPA and DHA have been shown to cause significant biochemical and physiological changes in the body that often have a positive influence on human nutrition and health. EPA and DHA can reduce serum and liver lipid levels due to the regulation of lipid metabolism.[33,34] Because linseed oil is rich in ALA (Table 2), a combination effect was found in linseed oil + SM supplementation in KK-A^y mice (Fig. 1 and 2). Compared with lard alone, linseed oil + SM supplementation could significantly reduce serum total cholesterol, non-HDL cholesterol, and LDL cholesterol (Fig. 1) and liver TL, NL, and cholesterol (Fig. 2).

SM supplementation reduced the intestinal lipid absorption in KK-A^y mice (Fig. 5). This might induce the decreasing tendency of hepatic TL (Fig. 2). Hepatic total fatty acids analyses also confirmed this effect of SM. Table 4 presents the significant decrease in hepatic total fatty acids of the lard-fed group with SM supplementation compared to mice without SM. The decrease in total fatty acids by SM supplementation was also found in the soybean oil- and linseed oil-fed mice, but the difference was not significant. In the soybean oil- and linseed oil-fed groups, SM supplementation increased long-chain PUFA such as ARA, EPA, DPA, and DHA (Table 4). The increase in EPA, DPA, and DHA, the active n-3 PUFA forms of ALA, may be related to the reduction of serum and liver lipid levels in the soybean oil- and linseed oil-fed KK-A^y mice supplemented with SM (Fig. 1 and 2). The increase in n-3 PUFA might be induced by the up-regulation of ALA bioconversion to EPA, DPA, and DHA; however, no change was observed in the related gene expressions, namely, Δ^6-desaturase (Fads2), elongase-5 (Elov5), Δ^5-desaturase (Fads1), and elongase-2 (Elov2) (Fig. 9). Another notable effect of SM supplementation was a decrease in liver 18:1n-9/18:0 and 16:1n-7/16:0 ratios in the soybean oil- and linseed oil-fed KK-A^y mice (Table 4). In our previous study (Watanabe *et al*. 2011), a significant decrease in 18:1n-9 was found in the liver lipids of the KK-A^y mice fed milk SL. The levels of 16:1n-7 and 18:1n-7 were also reduced by the SL feeding. Thus, the down-regulation of SCD1 by milk SL has been suggested. The present study demonstrates the reduction of the SCD1 gene expression by SM supplementation (Fig. 9A).

The different activity of SM on wild-type and obese/diabetic mice suggests the possibility that SM may be useful for the improvement of hyperlipidemia in subjects with metabolic disorders. The major mechanism for this effect will be the promotion of intestinal lipid secretion. On the other hand, further studies may be needed to investigate

the regulatory effect of dietary SM or its metabolites on lipid metabolism. The present study suggests the effect of SM on ALA and 18:0 bioconversion to longer chain n-3 PUFA and 18:1n-9, respectively. This might be in part related to the biological activity of SM. Longer chain n-3 PUFA from ALA are well-known to show hypolipidemic and/or hypocholesterolemic effects. In addition, studies in humans and animal models have revealed that modulation of SCD1 activity by dietary intervention or genetic manipulation strongly influences several facets of energy metabolism to affect susceptibility to obesity, insulin resistance, diabetes and hyperlipidemia.[35-37]

SM is not rapidly hydrolyzed in the intestines of rodents because of the low activity of rodent SM phosphodiesterase. In humans, the hydrolysis of SM is relatively faster and more efficient compared to rodents.[28,38] Dietary SL can be hydrolyzed to their components, such as sphingoid bases, fatty acids, and the polar head group, by intestinal enzymes and are then taken up by mucosal cells.[11] A large portion of sphingosine absorbed by the intestine is metabolized to fatty acids and a small part is resynthesized to complex sphingolipids. Therefore, more effort will be needed to investigate the direct action of SM metabolites, such as sphingoid bases, in biological systems.

In conclusion, our present study showed the inhibitory effect of SM on intestinal lipid absorption on obese/diabetic KK-A^y mice and wild-type C57BL/6J mice fed different types of dietary lipids. The reduction of lipid absorption by SM supplementation to KK-A^y mice induced serum and liver lipid decrease; however, this effect of SM was not found in wild-type C57BL/6J mice, suggesting the effectiveness of SM on subjects with metabolic disorders.

References

1. V. L. Spitsberg, Inivited review: bovine milk fat globule membrane as a potential nutraceuticals, *J. Dairy Sci.*, 2005, **88**, 2289-2294.
2. G. Contarini and M. Povolo, Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies, *Int. J. Mol. Sci.* 2013, **14**, 2808-2831.
3. E. Wat, S. Tandy, E. Kapera,A. Kamili, R. W. S. Chung, A. Brown, M. Rowney and J. S. Cohn, Dietary phospholipid-rich dairy milk extract reduces hepatomegaly, hepatic steatosis and hyperlipidemia in mice fed a high-fat diet, *Atherosclerosis* 2009, **205**, 144-150.
4. A. Kamili, E. Wat, R. W. S. Chung, S. Tandy, J. M. Weir, P. J. Meikle and J. S. Cohn, Hepatic accumulation of intestinal cholesterol is decreased and fecal cholesterol excretion is increased in mice fed a high-fat diet supplemented with milk

phospholipids, *Nutr. Metab.*, 2010, **7**, 90-101.
5. S. Watanabe, T. Takahashi, L. Tanaka, Y. Haruta, M. Shiota, M. Hosokawa and K. Miyashita, The effect of milk polar lipids separated from butter serum on the lipid levels in the liver and the plasma of obese-model mouse (KK-Ay), *J. Functional Foods* 2011, **3**, 313-320.
6. S. Keller, A. Malarski, C. Reuther, R. Kertscher, M. Kiehntopf and G. Jahreis, Milk phospholipid and plant sterol-dependent modulation of plasma lipids in healthy volunteers, *Eur. J. Nutr.*, 2013, **52**, 1169-1179.
7. J. C. Astaire, R. Ward, J. B. German and R. Jiménez-Flores, Concentration of polar MFGM lipids from buttermilk by microfiltration and supercritical fluid extraction, *J. Dairy Sci.*, 2003, **86**, 2297-2307.
8. L. Stillway and S. J. Harmon, A procedure for detecting phosphonolipids on chromatograrns, *J. Lipid Res.*, 1980, **21**, 1141-1143.
9. J. Folch, M. Lees and G. H. S. Stanley, A simple method for the isolation and purification of total lipides from animal tissues, *J. Biol. Chem.* 1957, **226**, 497-509.
10. E. Razmpoosh, M. Javadi, H.-S. Ejtahed and P. Mirmiran, Probiotics as beneficial agents in the management of diabetes mellitus: a systematic review, *Diabetes Metab. Res. Rev.* 2016, **32**, 143-168.
11. J. E. Bowe, Z. J. Franklin, A. C. Hauge-Evans, A. J. King, S. J. Persaud and P. M. Jones, Assessing glucose homeostasis in rodent models, *J. Endocrinol.*, 2014, **222**, G13-25.
12. A. F. Prevot and F. X. Mordret, Utilisation des colonnes capillaries de verre pour l'analyse des corps gras par chromotographie en phase gazeuse, *Rev. Fse. Corps. Grasl.*, 1976, **23**, 409-423.
13. R. -D. Duan and A. Nilsson, Metabolism of sphingolipids in the gut and its relation to inflammation and cancer development, *Prog. Lipid Res.*, 2009, **48**, 62-72.
14. P. J. Quinn, Lipid matrix model of membrane raft structure, *Prog. Lipid Res.*, 2010, **49**, 390-406.
15. B. M. Castro, M. Prieto L. C. Silva, A simple sphingolipid with unique biophysical properties, *Prog. Lipid Res.*, 2014, **54**, 53-67.
16. H. Vesper, E. M. Schmelz, M. N. Nikolova-Karakashian, D. L. Dillehay, D. V. Lynch and A. H. Merrill, Sphingolipids in food and the emerging importance of sphingolipids to nutrition, *J. Nutr.*, 1999, **129**, 1239-1250.
17. M. Lecomte, C. Bourlieu, E. Meugnier, A. Penhoat, D. Cheillan, G. Pineau, E. Loizon, M. Trauchesse, M. Claude, O. Ménard, A. Géloën, F. Laugerette and M. -C. Michalski, Milk polar l ipids affect in vitro digestive lipolysis and postprandial lipid

metabolism in mice, *J. Nutr.*, 2015, **145**, 1770-1777.
18. I. Duivenvoorden, P. J. Voshol, P. C. N. Rensen, W. van Duyvenvoorde, J. A. Romijn, J. J. Emeis, L. M. Havekes and W. F. Nieuwenhuizen, Dietary sphingolipids lower plasma cholesterol and triacylglycerol and prevent liver steatosis in APOE*3Leiden mice, *Am. J. Clin. Nutr.*, 2006, **84**, 312-321.
19. L. Nyberg, R. D. Duan and A. Nilsson, A mutual inhibitory effect on absorption of sphingomyelin and cholesterol, *J. Nutr. Biochem.*, 2000, **11**, 244-249.
20. E. R. M. Eckhardt, D. Q. –H. Wang, J. M. Donovan and M. C. Carey, Dietary shingomyelin suppresses intestinal cholesterol absorption by decreasing thermodynamic activity of cholesterol monomers, *Gastroenterology*, 2002, **122**, 948-956.
21. S. K. Noh and S. I. Koo, Egg sphingomyelin lowers the lymphatic absorption of cholesterol and α-tocopherol in rats, *J. Nutr.*, 2003, **133**, 3571-3576.
22. K. Yunoki, M. Renaguli, M. Kinoshita, H. Matsuyama, S. Mawatari, T. Fujino, Y. Kodama, M. Sugiyama and M. Ohnishi, Dietary sphingolipids ameliorate disorders of lipid metabolism in Zucker fatty rats, *J. Agric. Food Chem.*, 2010, **58**, 7030-7035.
23. V. R. Ramprasath, P. J. H. Jones, D. D. Buckley, L. A. Woollett and J. E. Heubi, Effect of dietary sphingomyelin on absorption and fractional synthetic rate of cholesterol and serum lipid profile in humans, *Lipids Health Dis.*, 2013, **12**, 125.
24. K. L. van Erpecum and M. C. Carey, Influence of bile salts on molecular interactions between sphingomyelin and cholesterol: relevance to bile formation and stability, *Biochim. Biophys. Acta*, 1997, **1345**, 269-282.
25. H. Chen, E. Born, S. N. Mathur, F. C. Jr. Johlin and F. J. Field, Sphingomyelin content of intestinal cell membranes regulates cholesterol absorption, *Biochem. J.*, 1992, **286**, 771-777.
26. A. Nilsson and R. D. Duan, Absorption and lipoprotein transport of sphingomyelin, *J. Lipid Res.*, 2006, **47**, 154-171.
27. L. Ohlsson, H. Burling and A. Nilsson, Long term effects on human plasma lipoproteins of a formulation enriched in butter milk polar lipid, *Lipids Health Dis.*, 2009, **8**, 44.
28. L. Ohlsson, H. Burling, R. D. Duan and A. Nilsson, Effects of a sphingolipid enriched dairy formulation on postprandial lipid concentrations, *Eur. J. Clin. Nutr.*, 2010, **64**, 1344-1349.
29. G. H. Norris, C. Jiang, J. Ryan, C. M. Porter and C. N. Blesso. Milk sphingomyelin improves lipid metabolism and alters gut microbiota in high fat diet-fed mice, *J. Nutr. Biochem.*, 2016, **30**, 93-101.

30. A. Baars, A. Oosting, E. Engels, D. Kegler, A. Kodde, L. Schipper, H. J. Verkade and E. M. van der Beek. Milk fat globule membrane coating of large lipid droplets in the diet of young mice prevents body fat accumulation in adulthood, Br. J. Nutr., 2016, **115**, 1930-1937
31. M. Lecomte, L. Couëdelo, E. Meugnier, P. Plaisancié, M. Létisse, B. Benoit, L. Gabert, A. Penhoat, A. Durand, G. Pineau, F. Joffre, A. Géloën, C. Vaysse, F. Laugerette and M. -C. Michalski, Dietary emulsifiers from milk and soybean differently impact adiposity and inflammation in association with modulation of colonic goblet cells in high-fat fed mice, *Mol. Nutr. Food Res.*, 2016, **60**, 609-620.
32. R. W. S. Chung, A. Kamili, S. Tandy, J. M. Weir, R. Gaire, G. Wong, P. J. Meikle, J. S. Cohn and K. -A. Rye, Dietary sphingomyelin lowers hepatic lipid levels and inhibits intestinal cholesterol absorption in high-fat-fed mice, *PLoS One*, 2013, **8**, e55949.
33. K. -B. Kim, Y. A. Nam, H. S. Kim, A. W. Hayes and B. -M. Lee, α-Linolenic acid: nutraceutical, pharmacological and toxicological evaluation, *Food Chem. Toxicol.*, 2014, **70**, 163-178.
34. T. A. Mori, Dietary n-3 PUFA and CVD: a review of the evidence, *Proc. Nutr. Soc.*, 2014, **73**, 57-64.
35. M. T. Flowers and J. M. Ntambi, Role of stearoyl-coenzyme A desaturase in regulating lipid metabolism, *Curr. Op. Lipid.*, 2008, **19**, 248-256.
36. M. T. Flowers and J. M. Ntambi, Stearoyl-CoA desaturase and its relation to high-carbohydrate diets and obesity, *Biochim. Biophys. Acta*, 2009, **1791**, 85-91.
37. C. M. Paton and J. M. Ntambi, Biochemical and physiological function of stearoyl-CoA desaturase, *Am. J. Physiol.*, 2008, **297**, E28-E37.
38. L. Nyberg, R. D. Duan, J. Axelson and A. Nilsson, Identification of an alkaline sphingomyelinase activity in human bile, *Biochim. Biophys. Acta*, 1996, **1300**, 42-48.

Figure legends

Figure 1. The effects of dietary lipids on serum lipid parameters of KK-A^y mice. (A), Total cholesterol; (B), NL; (C), PL; (D), non-HDL cholesterol; (E), LDL cholesterol; (F), HDL cholesterol. Values represent the means ± SEM of seven mice per group. A two-way ANOVA analysis showed that serum lipid parameters except for LDL cholesterol were not affected by the interaction of dietary lipids and SM feeding. Therefore, the significance was compared by Tukey's post hoc analysis except for LDL

cholesterol. Different letters (a, b, c) show significant differences at $P < 0.05$. The comparison of LDL cholesterol was done with one way ANOVA and t-test on two groups fed the same dietary lipid with or without SM ($^{\#}P < 0.05$ vs without SM) .

Figure 2. The effects of dietary lipids on liver lipid levels of KK-A^y mice. (A), TL; (B), NL; (C), PL; (D), TAG; (E), cholesterol. Values represent the means ± SEM of seven mice per group. A two-way ANOVA analysis showed that all lipid parameters were not affected by the interaction of dietary lipids and SM feeding. Therefore, the significance was compared by Tukey's post hoc analysis. Different letters (a, b, c) show significant differences at $P < 0.05$.

Figure 3. The effects of dietary lipids on serum lipid levels of C57BL/6J mice. (A), TL; (B), TAG; (C), cholesterol. Values represent the means ± SEM of six mice per group. A two-way ANOVA analysis showed that all lipid parameters were not affected by the interaction of dietary lipids and SM feeding. The analysis also showed no significant difference between the groups ($P < 0.05$).

Figure 4. The effects of dietary lipids on liver lipid levels of C57BL/6J mice. (A), TL; (B), NL; (C), PL; (D), TAG; (E), cholesterol. Values represent the means ± SEM of six mice per group. A two-way ANOVA analysis showed that all lipid parameters were not affected by the interaction of dietary lipids and SM feeding. The analysis also showed no significant difference between the groups ($P < 0.05$).

Figure 5. The effects of SM supplementation on fecal lipid levels of KK-A^y mice fed lard, soybean oil, and linseed oil. (A), TL; (B), cholesterol; (C), bile acid. Values represent the means ± SEM of seven mice per group. A two-way ANOVA analysis showed that all lipid parameters were significantly ($P < 0.05$) affected by the interaction of dietary lipids and SM feeding. Therefore, the comparison of lipid parameters were done with one way ANOVA and t-test on two groups fed the same dietary lipid with or without SM ($^{\#}P < 0.05$ vs without SM) .

Figure 6. The effects of SM supplementation on fecal TL levels of C57BL/6J mice fed linseed and fish oil after 1 day (A), 14 days (B), 27 days (C) of feeding. Values represent the means of six mice per group.

Figure 7. The effects of SM supplementation on fecal TL levels of C57BL/6J mice fed soybean oil after 15 days (A) and 29 days (B) of feeding. Values represent the means ± SEM of six mice per group. The comparison was done with one way ANOVA and t-test on two groups with or without SM ($^{\#}P$ <0.05 vs without SM) .

Figure 8. The gene expressions of the liver associated with cholesterol metabolism in KK-A^y mice fed different dietary lipids. Values represent the means ± SEM of seven mice per group. A two-way ANOVA analysis showed that all lipid parameters were not affected by the interaction of dietary lipids and SM feeding. The analysis also showed no significant difference between the groups (P <0.05).

Figure 9. The gene expressions of the liver associated with the bioconversion of ALA to DHA in KK-A^y mice fed different dietary lipids. Values represent the means ± SEM of seven mice per group. A two-way ANOVA analysis showed that all lipid parameters were not affected by the interaction of dietary lipids and SM feeding. Therefore, the significance was compared by Tukey's post hoc analysis. Different letters (a, b, c) show significant differences at $P < 0.05$.

Table 1. Composition (g/kg) of experimental diets

	KK-*Ay* mice						C57BL/6J mice					
Diet ingredient	Lard	Lard +SM	Soybean oil	Soybean oil + SM	Linseed oil	Linseed oil + SM	Linseed oil	Linseed oil + SM	Fish oil	Fish oil + SM	Lard + Soybean oil	Lard + Soybean oil +SM
Corn starch	397.49	397.49	397.49	397.49	397.49	397.49	397.49	397.49	397.49	397.49	157.1	157.1
Dextrinized corn starch	132	132	132	132	132	132	132	132	132	132	52.4	52.4
Casein	200	200	200	200	200	200	200	200	200	200	260	260
Sucrose	100	100	100	100	100	100	100	100	100	100	130	130
Cellulose (KC flock)	50	50	50	50	50	50	50	50	50	50	50	50
AIN93G mineral mix	35	35	35	35	35	35	35	35	35	35	35	35
AIN93G vitamin mix	10	10	10	10	10	10	10	10	10	10	10	10
L-cystine	3	3	3	3	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
t-Butylhydroquinoe	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.02	0.014
Lard	70	60	0	0	0	0	0	0	0	0	230	230
Soybean oil	0	0	70	60	0	0	0	0	0	0	70	60
Linseed oil	0	0	0	0	70	60	70	60	0	0	0	0
Fish oil	0	0	0	0	0	0	0	0	70	60	0	0
SPM	0	10	0	10	0	10	0	10	0	10	0	10

Table 1. Composition (g/kg) of experimental diets

	KK-*Ay* mice						C57BL/6J mice					
Fatty acid	Lard	Lard +SM	Soybean oil	Soybean oil + SM	Linseed oil	Linseed oil + SM	Linseed oil	Linseed oil + SM	Fish oil	Fish oil + SM	Lard + Soybean oil	Lard + Soybean oil +SM
16:0	23.74	24.43	9.91	10.73	5.24	5.95	5.32	6.11	8.16	8.92	21.94	21.94
18:0	14.08	13.58	3.84	3.90	3.29	3.26	3.80	3.83	2.56	2.57	11.79	11.49
20:0	ND*	0.03	0.35	0.38	0.14	0.16	0.15	0.17	0.29	0.32	ND*	ND*
22:0	ND*	1.37	0.45	1.56	0.13	1.28	0.18	1.33	0.19	1.61	ND*	0.27
23:0	ND*	2.18	ND*	2.08	ND*	1.87	ND*	1.88	ND*	2.29	ND*	0.37
24:0	ND*	1.39	0.16	1.43	ND*	1.04	ND*	1.25	0.07	1.53	ND*	0.26
16:1n-7	2.39	2.14	0.10	0.10	0.06	0.06	0.08	0.05	2.53	2.34	2.02	2.10
18:1n-7	2.49	2.51	1.49	1.34	0.56	0.59	0.68	0.67	1.30	1.22	2.14	2.80
18:1n-9	42.01	37.46	25.70	20.38	18.75	17.86	20.43	19.66	4.81	4.53	38.33	37.14
18:2n-6	9.10	8.63	50.38	49.48	15.94	15.20	15.41	14.54	0.68	0.60	18.84	16.91
18:3n-3	0.62	0.48	5.72	6.52	53.13	49.91	51.21	48.33	0.46	0.47	1.50	1.34
18:4n-3	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	2.30	2.10	ND*	ND*
20:4n-6	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	2.47	2.26	ND*	ND*
20:5n-3	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	17.75	16.39	ND*	ND*
22:5n-3	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	3.35	3.19	ND*	ND*
22:6n-3	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	33.82	32.02	ND*	ND*

*Not detected.

Table 2. Major fatty acid composition (wt% of total fatty acids) of dietary lipids

	KK-A^y mice						C57BL/6J mice			
		Lard		Soybean oil		Linseed oil		Linseed oil		Fish oil
Fatty acid	Lard	+SM	Soybean oil	+ SM	Linseed oil	+ SM	Linseed oil	+ SM	Fish oil	+ SM
16:0	23.74	24.43	9.91	10.73	5.24	5.95	5.32	6.11	8.16	8.92
18:0	14.08	13.58	3.84	3.90	3.29	3.26	3.80	3.83	2.56	2.57
20:0	ND*	0.03	0.35	0.38	0.14	0.16	0.15	0.17	0.29	0.32
22:0	ND*	1.37	0.45	1.56	0.13	1.28	0.18	1.33	0.19	1.61
23:0	ND*	2.18	ND*	2.08	ND*	1.87	ND*	1.88	ND*	2.29
24:0	ND*	1.39	0.16	1.43	ND*	1.04	ND*	1.25	0.07	1.53
16:1n-7	2.39	2.14	0.10	0.10	0.06	0.06	0.08	0.05	2.53	2.34
18:1n-7	2.49	2.51	1.49	1.34	0.56	0.59	0.68	0.67	1.30	1.22
18:1n-9	42.01	37.46	25.70	20.38	18.75	17.86	20.43	19.66	4.81	4.53
18:2n-6	9.10	8.63	50.38	49.48	15.94	15.20	15.41	14.54	0.68	0.60
18:3n-3	0.62	0.48*	5.72	6.52*	53.13	49.91*	51.21	48.33*	0.46	0.47
18:4n-3	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	2.30	2.10
20:4n-6	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	2.47	2.26
20:5n-3	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	17.75	16.39
22:5n-3	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*	3.35	3.19
22:6n-3	ND*	ND	ND*	ND	ND*	ND	ND*	ND	33.82	32.02

*Not detected.

Table 3. Body weight, food intake, water intake, tissue weight, and plasma lipids

KK-A^y mice[1)]	Lard	Lard + SM	Soybean oil	Soybean oil + SM	Linseed oil	Linseed oil + SM
Final body weight (g)	40.58±1.06	38.93±1.50	39.65±0.84	37.06±0.36	41.01±1.24	38.91±0.46
Food intake (g/day)	5.57±0.30	4.60±0.42	5.02±0.39	4.47±0.20	5.62±0.26	5.75±0.22
Water intake (g/day)	43.90±1.42^{a}	40.67±3.14^{a,b}	33.50±1.72^{b}	36.21±1.24^{a,b}	34.67±2.52^{b}	34.27±1.63^{b}
Liver weight (g/100g BW)	6.48±0.18^{a}	6.45±0.24^{a,b}	5.73±0.17^{a,b}	5.65±0.15^{a,b}	5.81±0.23^{a,b}	5.62±0.22^{b}
Kidney weight (g/100g BW)	1.63±0.06	1.52±0.03	1.54±0.05	1.53±0.06	1.68±0.08	1.72±0.05
Spleen weight (g/100g BW)	0.25±0.01	0.31±0.02	0.31±0.03	0.33±0.32	0.26±0.01	0.31±0.03
Large intestine weight (g/100g BW)	0.80±0.08	0.66±0.07	0.70±0.08	0.79±0.07	0.70±0.06	0.76±0.06
Small intestine weight (g/100g BW)	3.17±0.25	3.13±0.20	2.88±0.13	3.21±0.16	3.39±0.21	3.16±0.11
Heart weight (g/100g BW)	0.44±0.03	0.40±0.02	0.47±0.02	0.42±0.01	0.47±0.01	0.45±0.02
Total WAT weight (g/100g BW)	11.26±0.41	11.53±0.27	11.47±0.39	10.32±0.28	10.68±0.33	10.08±0.32
Blood glucose (mg/dL)	723.29±28.35	673.17±45.14	650.25±42.00	683.00±41.58	730.83±21.75	606.14±18.69

C57BL/6J mice[2)]	Linseed oil	Linseed oil + SM	Fish oil	Fish oil + SM
Final body weight (g)	26.27±0.50	24.59±0.31	24.17±0.27	24.21±0.86
Food intake (g/day)	3.24±0.05^{a}	3.09±0.05^{a,b}	2.89±0.04^{b}	2.95±0.04^{b}
Water intake (g/day)	5.00±0.10^{a}	3.71±0.07^{b}	4.14±0.07^{b,c}	4.30±0.06^{c}
Liver weight (g/100g BW)	4.72±0.08^{a,b}	4.51±0.08^{a}	5.23±0.05^{b}	5.03±0.09^{a,b}
Kidney weight (g/100g BW)	1.94±0.04	1.94±0.03	2.00±0.03	1.99±0.04
Spleen weight (g/100g BW)	1.03±0.01^{a}	1.03±0.02^{a}	1.17±0.02^{b}	1.17±0.01^{b}
Large intestine weight (g/100g BW)	1.56±0.06	1.69±0.05	1.56±0.06	1.73±0.15
Small intestine weight (g/100g BW)	4.13±0.11^{a}	4.11±0.07^{a}	4.61±0.32^{a,b}	4.89±0.12^{b}
Heart weight (g/100g BW)	1.20±0.03	1.24±0.03	1.24±0.02	1.26±0.02
Total WAT weight (g/100g BW)	5.95±0.24	7.25±0.53	6.38±0.35	6.00±0.20
Blood glucose (mg/dL)	75.17±3.57	80.67±3.38	71.17±2.57	70.67±3.80

[1)]n=7

[2)]n=6

^{a,b,c}A two-way ANOVA analysis showed that all data were not affected by the interaction of dietary lipids and SM feeding, then, the comparison was done by Tukey's post hoc analysis. Different letters show significantly different at $P < 0.05$.

Table 4. Effect of dietary lipids and SPM on fatty acid content in liver of KK-A^y mice (n=7)

	Lard	Lard + SM	Soybean oil	Soybean oil + SM	Linseed oil	Linseed oil + SM
Fatty acid (mg/1g tissue)						
16:0	26.82±2.89	11.34±1.34 #	12.66±3.25	11.37±1.05	11.28±0.98	7.85±0.51#
18:0	5.05±0.62	2.79±0.20 #	4.49±0.34	4.34±0.43	3.66±0.26	3.92±0.25#
16:1n-7	4.84±0.64	2.08±0.28 #	2.20±0.30	1.24±0.19 #	1.71±0.20	0.77±0.11#
18:1n-7	5.35±0.73	2.90±0.34 #	2.06±0.23	1.19±0.11 #	1.12±0.10	0.58±0.09#
18:1n-9	37.25±5.36	19.51±2.42 #	15.24±2.06	8.37±1.01 #	13.39±1.40	7.28±1.09#
18:2n-6	4.92±0.54 a	2.31±0.49 a	11.19±0.80 b	8.00±0.79 c	4.57±0.37 a	3.88±0.28 a
18:3n-3	ND *	ND *	0.47±0.05	0.33±0.04	4.68±0.51	3.14±0.39#
20:4n-6	4.14±0.54	2.64±0.12 #	3.71±0.27	4.11±0.40	0.84±0.06	1.09±0.06
20:5n-3	ND *	0.06±0.01 #	ND *	0.27±0.03 a	2.20±0.14 b	2.76±0.22 b
22:5n-3	ND *	0.09±0.01 #	ND *	0.41±0.03 #	0.52±0.04	1.03±0.09#
22:6n-3	1.71±0.21	1.47±0.07	1.91±0.11	2.79±0.26 #	1.41±0.12	2.04±0.15#
Total fatty acids	89.84±10.78	45.19±4.84 #	53.93±4.34	42.42±3.97	45.37±3.48	28.59±6.79#
Total n-6 fatty acids	9.06±1.05 a,b	4.94±0.51 c	14.90±0.95 d	12.11±1.16 a,d	5.40±0.42 b,c	4.98±0.28 c
Total n-3 fatty acids	1.71±0.20 a	1.62±0.07 a	2.38+0.12 a,b	3.80±0.34 b	8.81±0.73 c	8.52±0.78 c
Ratio of each fatty acid						
18:1n-9/18:0	7.63±0.91 a	6.92±0.67 a	3.47±0.51 b	1.92±0.11 b	3.75±0.43 b	1.98±0.21 b
16:1n-7/16:0	0.18±0.01	0.18±0.01	0.18±0.02	0.11±0.01 #	0.15±0.01	0.10±0.01#

a,b,c A two-way ANOVA analysis showed that some of the fatty acid data were affected by the interaction of dietary lipids and SM feeding, but some of them were not affected. When no interaction was present, the significance was compared by Tukey's post hoc analysis. Different letters (a, b, c) show significant differences at $P < 0.05$.

If an interaction was present, one-way ANOVA and t-test were performed between two groups fed the same dietary lipid with or without SM (#P < 0.05 *vs* without SM).

∗Not detected.

Table 5. Effect of dietary lipids and SPM on fatty acid content in liver of C57BL/6J mice (n=6)

	Linseed oil	Linseed oil + SM	Fish oil	Fish oil + SM
Fatty acid (mg/1g tissue)				
16:0	9.30±072[a]	12.24±0.75[a,b]	12.56±0.88[a,b]	17.48±2.06[b]
18:0	2.55±0.16	2.65±0.06	2.64±0.14	3.14±0.17
16:1n-7	1.96±0.29	3.04±0.38	1.43±0.22	2.39±0.45
18:1n-7	0.46±0.04[a,b]	0.59±0.04[a]	0.30±0.02[b]	0.39±0.05[a,b]
18:1n-9	9.52±1.08[a,b]	11.98±0.58[a]	5.80±0.32[b]	8.17±120[a,b]
18:2n-6	7.23±0.62[a,b]	8.53±0.36[a]	2.80±0.21[c]	3.64±0.59[b,c]
18:3n-3	8.23±1.21[a]	10.72±0.68[a]	0.15±0.04[b]	0.19±0.04[b]
20:4n-6	0.88±0.034[a]	0.94±0.03[a]	1.58±0.08[b]	1.63±0.11[b]
20:5n-3	1.41±0.08	1.51±0.08	1.37±0.18	1.88±0.293
22:5n-3	0.55±0.04[a]	0.67±0.04[a,b]	0.74±0.04[a,b]	0.97±0.13[b]
22:6n-3	2.27±0.13[a]	2.31±0.06[a]	9.55±0.51[b]	12.37±1.41[c]
Total fatty acids	44.36±4.20	55.17±2.66	38.92±2.23	52.24±6.40
Total n-6 fatty acids	9.11±1.22[a]	11.66±0.70[a]	1.73±0.10[b]	1.82±0.14[b]
Total n-3 fatty acids	12.46±1.41	15.21±0.82	11.81±0.71	15.42±1.85
Ratio of each fatty acid				
18:1n-9/18:0	3.72±0.30[a]	4.52±0.23[a]	2.22±0.15[b]	2.55±0.22[b]
16:1n-7/16:0	0.21±0.02[a]	0.24±0.02[a]	0.11±0.01[b]	0.13±0.01[b]

[a,b,c]A two-way ANOVA analysis showed that all data were not affected by the interaction of dietary lipids and SM feeding, then, the comparison was done by Tukey's post hoc analysis. Different letters show significantly different at $P < 0.05$.

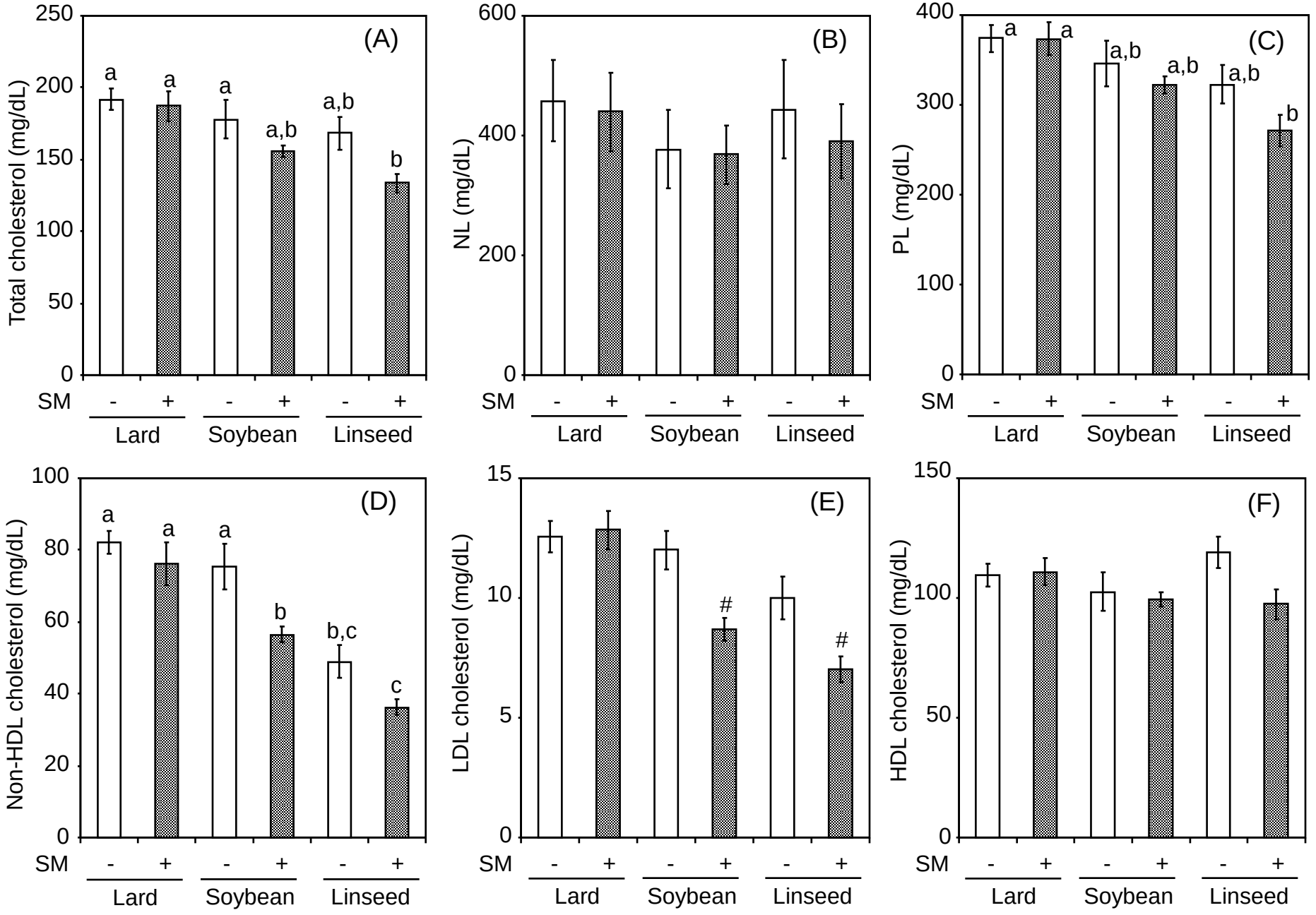
(A)
Total cholesterol (mg/dL)
250
200
150
100
50
0
a
a
a
a,b
a,b
b
SM
-
+
-
+
-
+
Lard
Soybean
Linseed
(B)
NL (mg/dL)
600
400
200
0
(C)
PL (mg/dL)
400
300
200
100
0
a
a
a,b
a,b
a,b
b
(D)
Non-HDL cholesterol (mg/dL)
100
80
60
40
20
0
a
a
a
b
b,c
c
(E)
LDL cholesterol (mg/dL)
15
10
5
0
#
#
(F)
HDL cholesterol (mg/dL)
150
100
50
0

Fig. 1

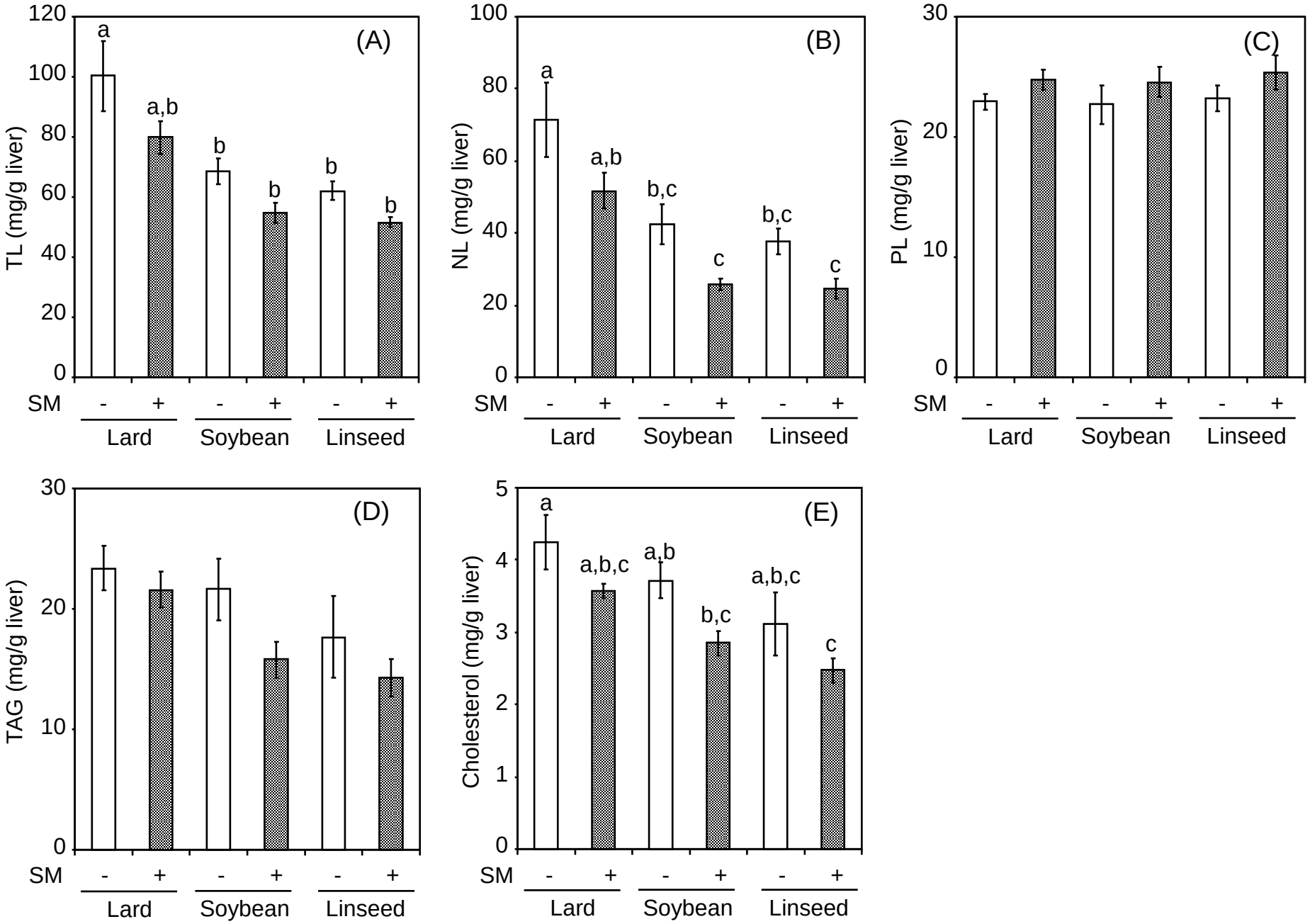
(A)
TL (mg/g liver)
120
100
80
60
40
20
0
a
a,b
b
b
b
b
SM - + - + - +
Lard
Soybean
Linseed
(B)
NL (mg/g liver)
100
80
60
40
20
0
a
a,b
b,c
c
b,c
c
SM - + - + - +
Lard
Soybean
Linseed
(C)
PL (mg/g liver)
30
20
10
0
SM - + - + - +
Lard
Soybean
Linseed
(D)
TAG (mg/g liver)
30
20
10
0
SM - + - + - +
Lard
Soybean
Linseed
(E)
Cholesterol (mg/g liver)
5
4
3
2
1
0
a
a,b,c
a,b
b,c
a,b,c
c
SM - + - + - +
Lard
Soybean
Linseed

Fig. 2

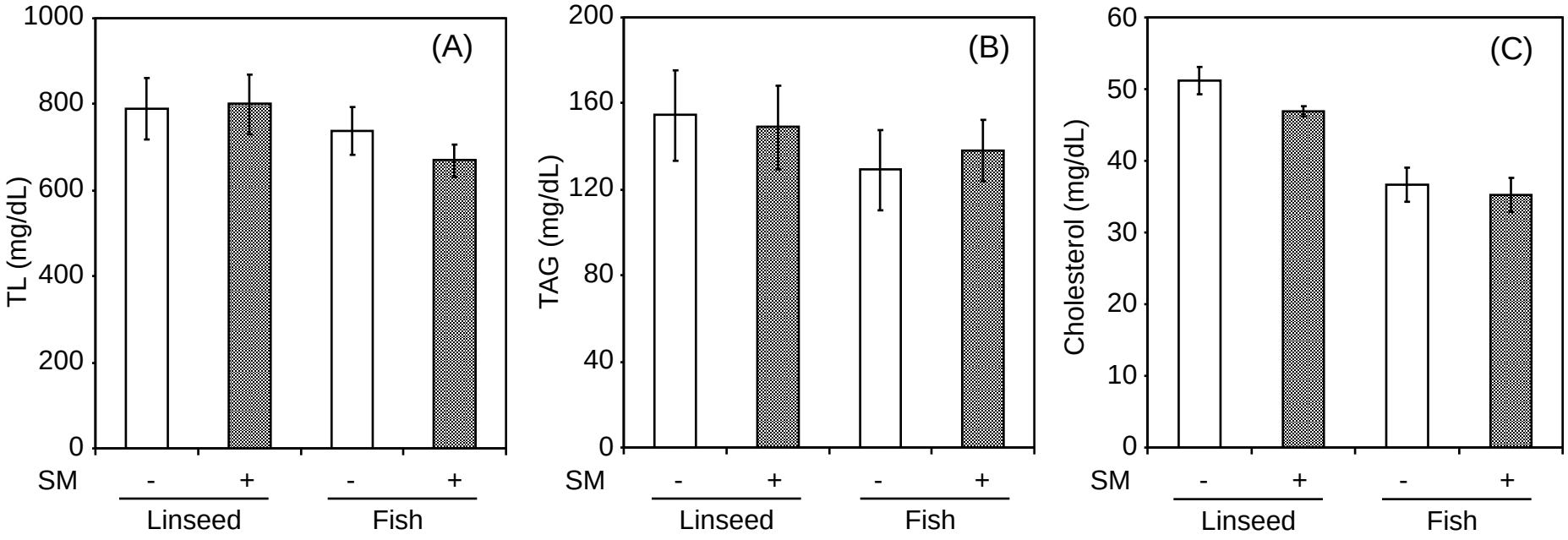
(A)
TL (mg/dL)
1000
800
600
400
200
0
SM
-
+
-
+
Linseed
Fish
(B)
TAG (mg/dL)
200
160
120
80
40
0
SM
-
+
-
+
Linseed
Fish
(C)
Cholesterol (mg/dL)
60
50
40
30
20
10
0
SM
-
+
-
+
Linseed
Fish

Fig. 3

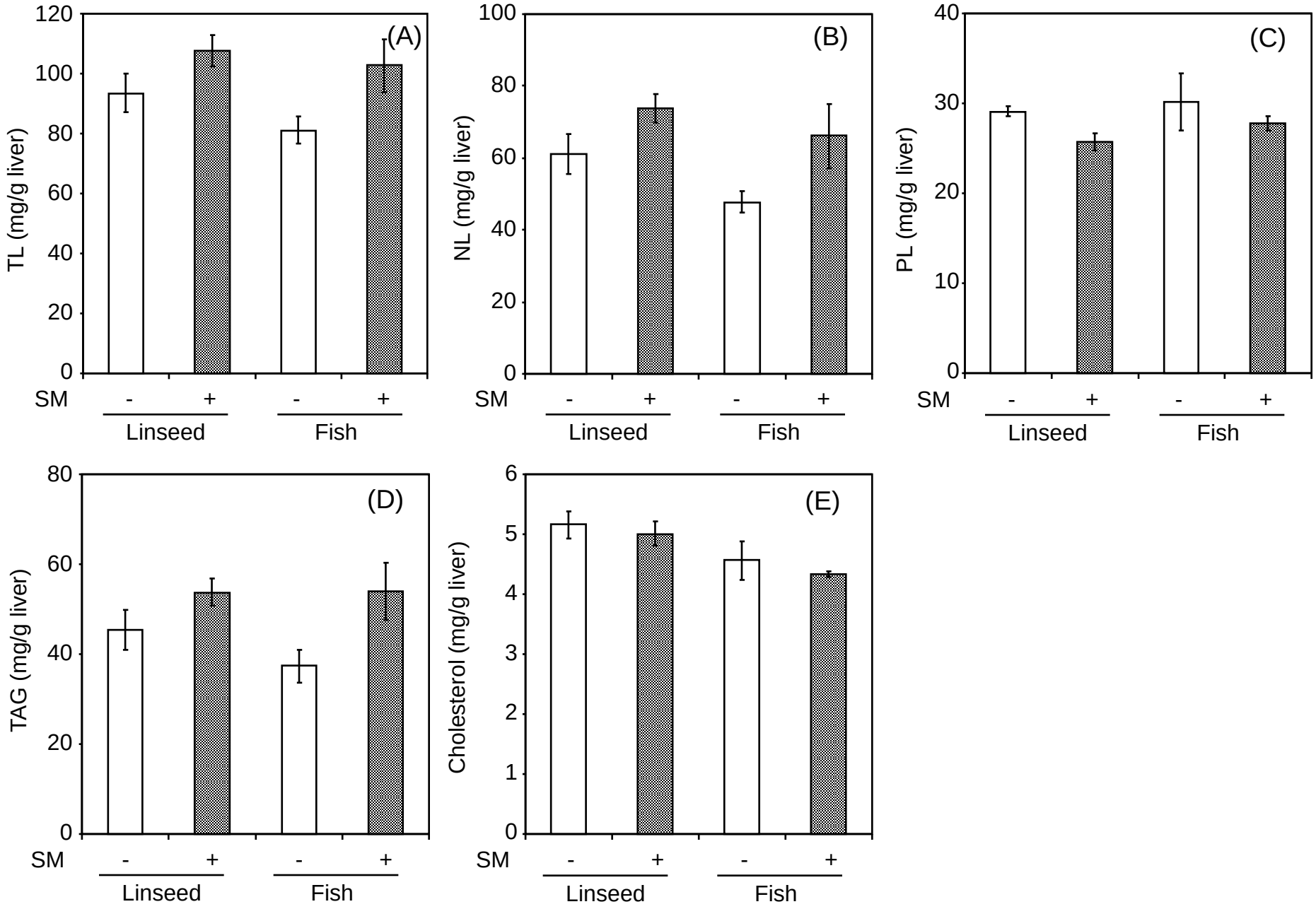
(A)
TL (mg/g liver)
120
100
80
60
40
20
0
SM
-
+
-
+
Linseed
Fish
(B)
NL (mg/g liver)
100
80
60
40
20
0
(C)
PL (mg/g liver)
40
30
20
10
0
(D)
TAG (mg/g liver)
80
60
40
20
0
(E)
Cholesterol (mg/g liver)
6
5
4
3
2
1
0

Fig. 4

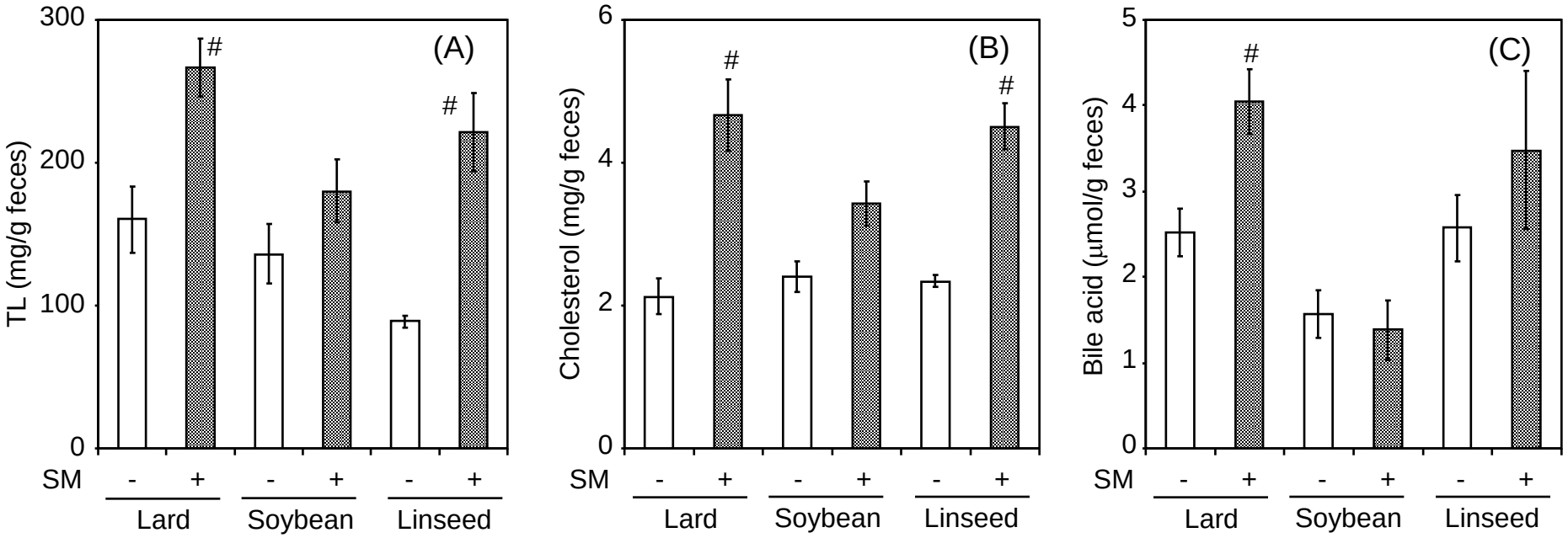
(A)
TL (mg/g feces)
300
200
100
0
#
#
SM
-
+
-
+
-
+
Lard
Soybean
Linseed
(B)
Cholesterol (mg/g feces)
6
4
2
0
#
#
SM
-
+
-
+
-
+
Lard
Soybean
Linseed
(C)
Bile acid (μmol/g feces)
5
4
3
2
1
0
#
SM
-
+
-
+
-
+
Lard
Soybean
Linseed

Fig. 5

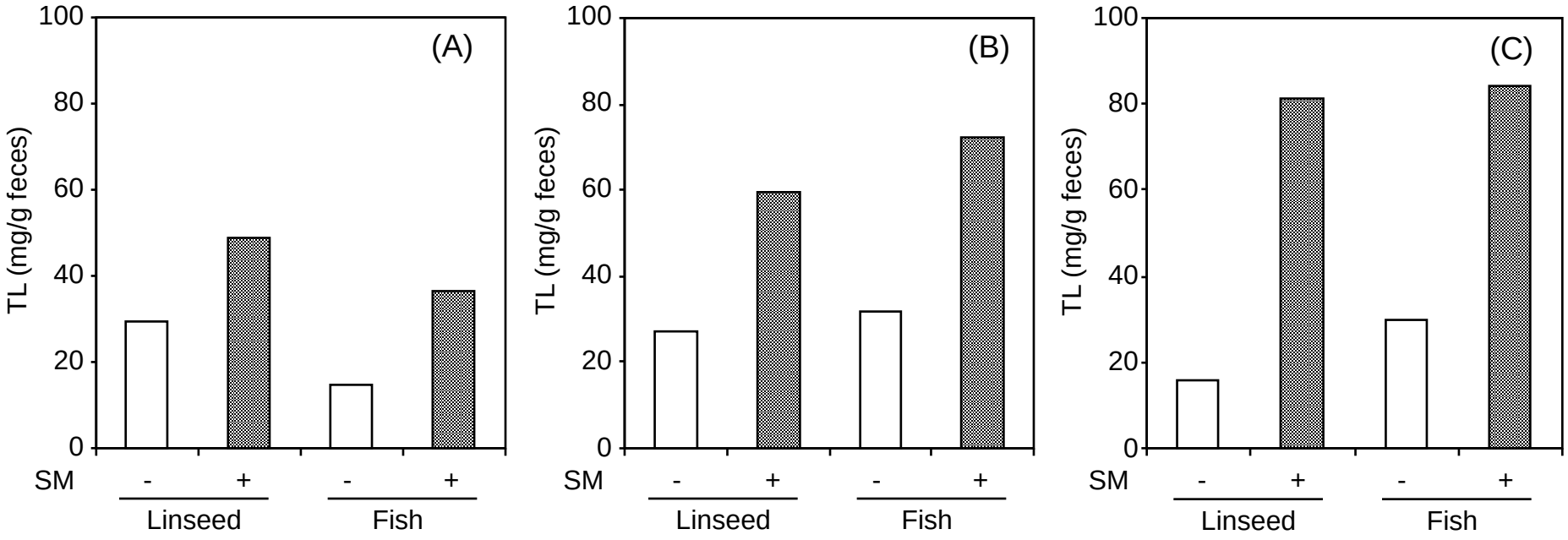

Fig. 6

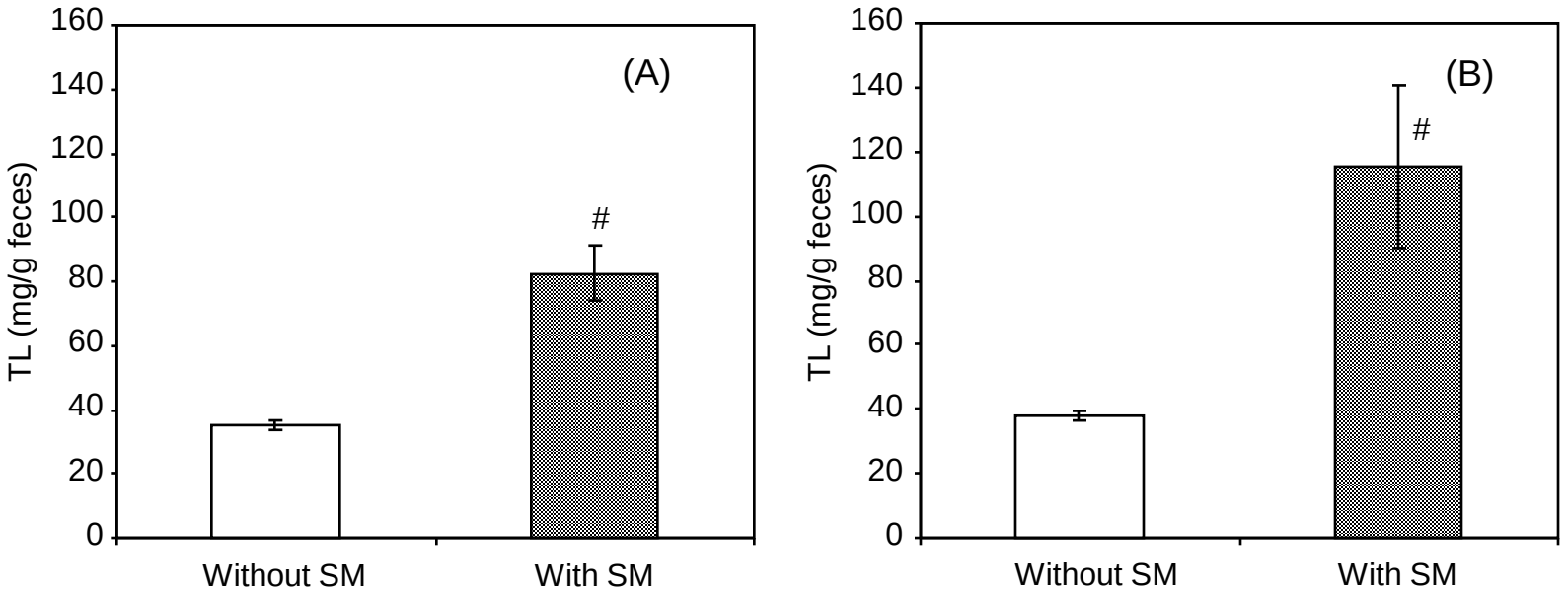
(A)
TL (mg/g feces)
160
140
120
100
80
60
40
20
0
#
Without SM
With SM
(B)
TL (mg/g feces)
160
140
120
100
80
60
40
20
0
#
Without SM
With SM

Fig. 7

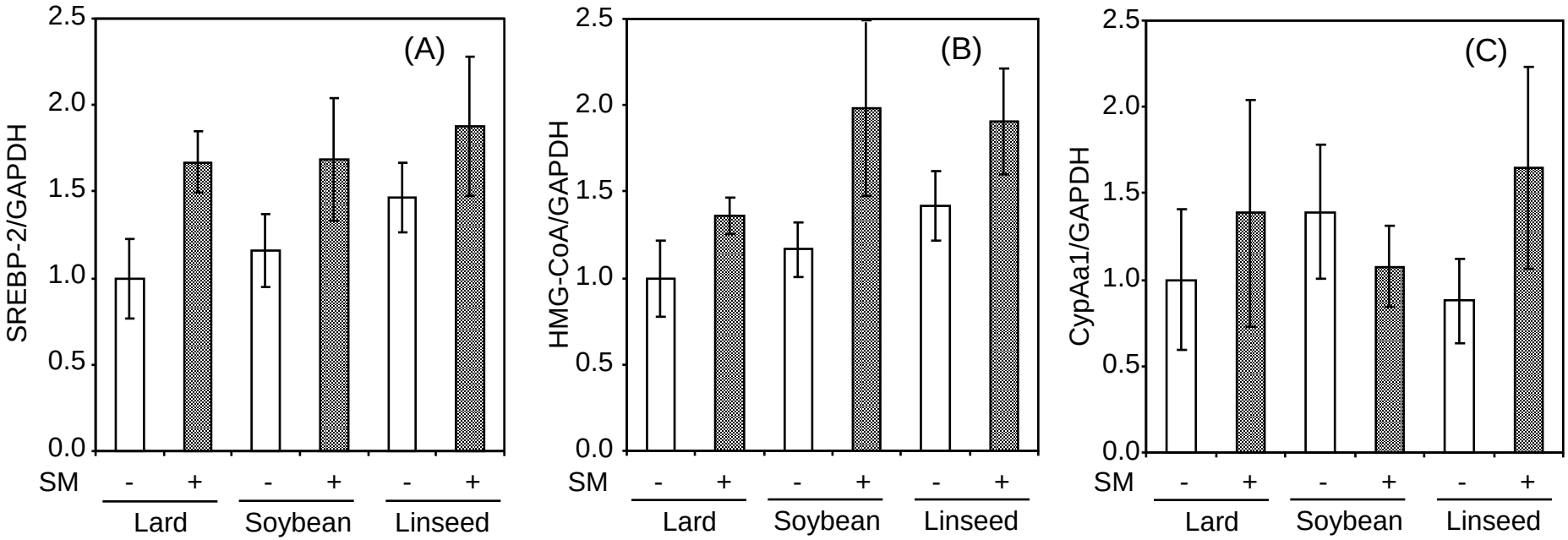
(A)
SREBP-2/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
SM
-
+
-
+
-
+
Lard
Soybean
Linseed
(B)
HMG-CoA/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
SM
-
+
-
+
-
+
Lard
Soybean
Linseed
(C)
CypAa1/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
SM
-
+
-
+
-
+
Lard
Soybean
Linseed

Fig. 8

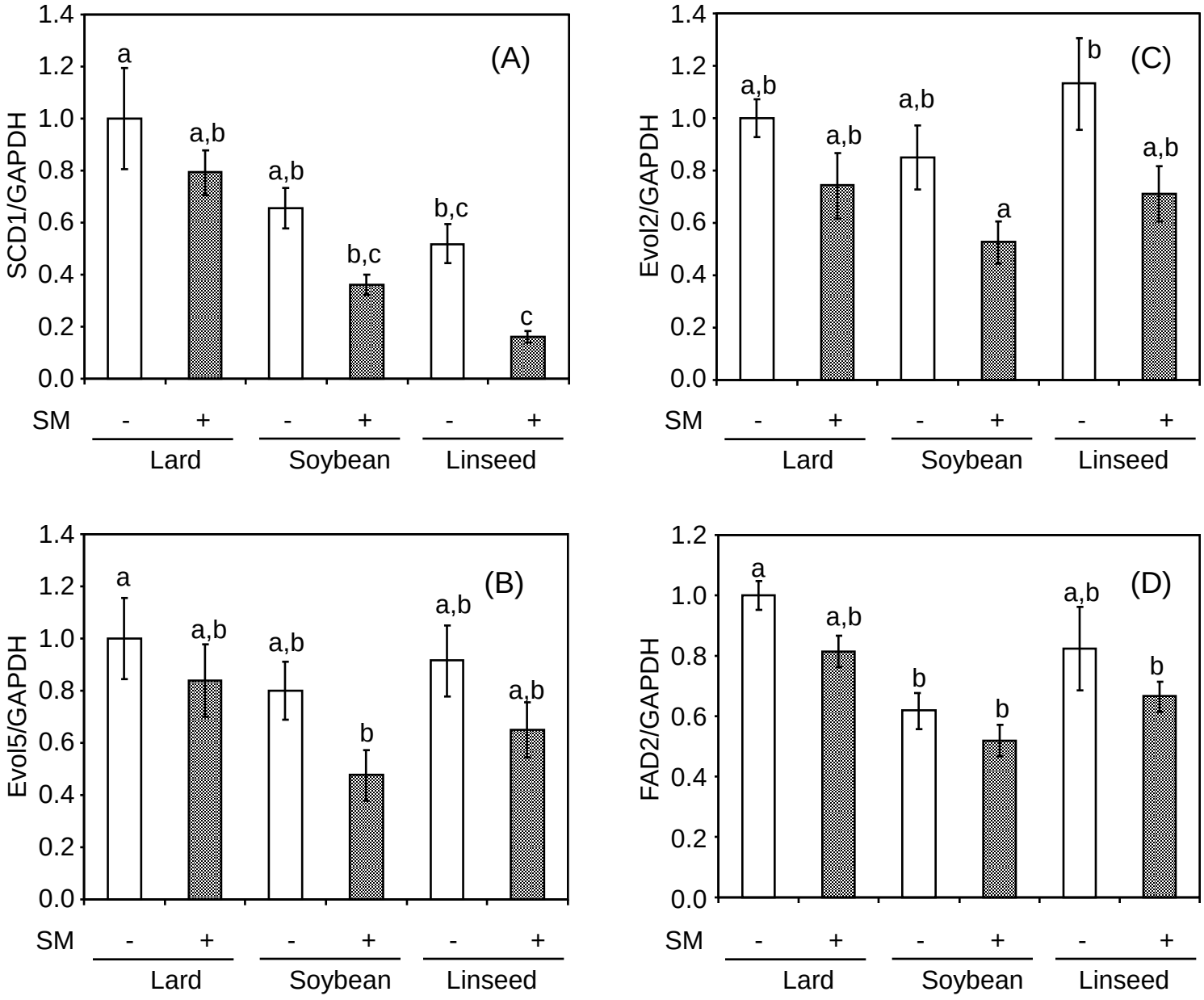
(A)
SCD1/GAPDH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a,b
a,b
b,c
b,c
c
SM
-
+
-
+
-
+
Lard
Soybean
Linseed
(C)
Evol2/GAPDH
a,b
a,b
a,b
a
b
a,b
(B)
Evol5/GAPDH
a
a,b
a,b
b
a,b
a,b
(D)
FAD2/GAPDH
a
a,b
b
b
a,b
b

Fig. 9