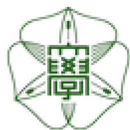
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The dietary effect of milk sphingomyelin on the lipid metabolism of obese/diabetes KK-A^y mice and wild-type C57BL/6J mice

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Running title: The effect of milk sphingomyelin

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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

37 phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine, and
38 phosphatidylinositol, while the major sphingolipids (SL) are glucosylceramide,
39 lactosylceramide, and sphingomyelin (SM).
40 Milk nutrients have attracted attention as functional foods and nutraceuticals with
41 potentially important cardioprotective properties. Recent studies showed that increased
42 consumption of milk and dairy products is associated with a reduced incidence of
43 obesity, insulin resistance, dyslipidemia, and type 2 diabetes, which are cardiovascular
44 risk factors.[2] Among the milk component, polar lipids rich in butter serum have been
45 considered active components in the improvement of lipid metabolism. The
46 hypolipidemic and/or hypocholesterolemic activity of milk PL and SL have been found
47 in animal models.[3-5] Wat *et al.*[5] reported a significant decrease in the liver weight, total
48 liver lipid, liver triacylglycerol (TAG), and total cholesterol and serum lipids of mice
49 fed a high-fat diet with a PL-rich dairy milk extract. Milk PL supplementation could
50 also significantly decrease the total liver cholesterol and TAG levels of mice (C57BL/6)
51 fed a high-fat diet.[4] On the other hand, we have found a significant decrease in the
52 plasma cholesterol, hepatic total cholesterol and TAG levels of obese/diabetic mice
53 (KK-A^y) by the supplementation of lipid concentrated butter serum (LC-BS).
54 Furthermore, when ceramide, SM, PE, and PC rich fractions from LC-BS was given to
55 the KK-Ay mice, significant decrease in plasma cholesterol and hepatic lipid levels was
56 found in the animals fed ceramide fraction. [5] The decrease was also found in the mice
57 fed SM fraction. On the other hand, there was little effect of PE and PC fractions on the
58 lipid levels. This result suggested that the effect of LC-BS is mainly due to SL, the main
59 components of LC-BS.[5] However, purity of ceramide and SM fractions used in the
60 study was 70.7 and 49.9% respectively. A human study has also demonstrated the
61 possible effect of the intake of milk polar lipids on cholesterol absorption from the
62 intestine and/or hepatic metabolism.[6]
63 Although several studies have demonstrated the hypolipidemic and
64 hypocholesterolemic activity of milk polar lipids, there has been no study on the effect
65 of purified lipid classes from milk polar lipids. Moreover, the mechanism for the
66 activity of the milk polar lipid on lipid metabolism has not been made clear. Thus, in the
67 present study, we separated SM from LC-BS and measured its dietary effect on serum,
68 liver, and fecal lipid contents by using obese/diabetic KK-A^y mice and wild-type
69 C57BL/6J mice.
70
71 **Materials and Methods**
72 **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

109 high-performance liquid chromatography (HPLC). The lipid fraction was dissolved in
110 chloroform-methanol-water (65:25:4, v/v/v) solution and analyzed by TLC as described
111 above. The spot was visualized by spraying with the Dittmer reagent, followed by
112 charring all spots at 150°C.
113 The purity of SM was also analyzed using HPLC. HPLC was performed with a
114 Shimadzu HPLC system (Shimadzu Seisakusho, Kyoto, Japan) equipped with a pump
115 (Shimadzu LC-20AD) and an evaporated light scattering detector (Hitachi ELSD-LT II).
116 The analysis was performed on a silica column (Mighttysil Si 60, 250 x 4.6 mm i.d;
117 Kanto Chemical Co. Ltd., Tokyo, Japan). The mobile phase consisted of
118 dichloromethane (A) and methanol/water (95:5, v/v) (B). A gradient elution procedure
119 was programmed as follows: 0-5 min, 99:1 (A:B, v/v); 20-25 min, 80:20 (A:B, v/v);
120 35-40 min, 10:90 (A:B, v/v); and 40-45 min, 1:99 (A:B, v/v). A linearly programmed
121 gradient went from 99:1 (A:B, v/v) to 80:20 (A:B, v/v) at 5-20 min and from 80:20
122 (A:B, v/v) to 10:90 (A:B, v/v) at 25-35 min, respectively. The flow rate was kept at 1.0
123 mL/min and the column temperature was maintained at 40°C. Each sample (*ca.* 10 mg)
124 was dissolved in dichloromethane/methanol (1:1, v/v) and 1 μl was injected onto HPLC.
125 The drift tube temperature was 50°C and the nebulizer gas (N_2) pressure was 350 kPa.
126 Milk SM was used as the standard.
127

128 **Animals and diets**

129 Obese/diabetic KK-A^y mice (male, four weeks old) and wild-type C57BL/6J mice (male,
130 four weeks old) were obtained from Japan CREA Co., Tokyo, Japan. The KK-A^y mice
131 were housed individually, while C57BL/6J mice of the same experimental group (n=6)
132 were housed in one cage. They had free access to food and tap water. Room temperature
133 and humidity were controlled at 23 ± 1˚C and 40-60% with a 12 h light/12 h dark cycle.
134 After acclimation for a week, including being fed a normal rodent diet MF (Oriental
135 Yeast Co., Ltd., Tokyo, Japan), the mice were randomly divided into groups of seven
136 (KK-A^y mice) or six (C57BL/6J mice) and were then fed experimental diets for four
137 weeks. The body weight, diet and water intake of each mouse was recorded daily. The
138 composition of the diets is shown in Table 1. Several studies[3-5] examined the effect of
139 milk polar lipids mainly rich in PL. These lipids also contained significant amounts of
140 SM and the SM level was from 0.25 - 0.55 wt% of total diet. The lipid content of SM in
141 the dietary lipids has been up to 12% in these studies. To make clear the dietary effect of
142 SM, 1.0% of SM (14% of the dietary lipids) was adapted in the present study.
143

144 **Ethics**

145 The research project was approved by the Ethical Committee at Hokkaido University
146 and all procedures for the use and care of animals for this research were carried out
147 under the approval by the Ethical Committee of Experimental Animal Care at Hokkaido
148 University.
149
150 **Fatty acid composition of dietary lipids**
151 Dietary lipids, lard, soybean oil, linseed oil, and fish oil, were obtained from Showa
152 Chemical Industry Co. Ltd., Tokyo, Wako Pure Chemical Ind., Osaka, Summit oil Mill
153 Co. Ltd., Chiba, Junsei Chemical Co. Inc., Tokyo, and Maruha Nichiro Co., Tsukuba,
154 Japan, respectively. After the dietary lipids were mixed with other dietary ingredients
155 (Table 1), the lipids were extracted from the diets with chloroform/methanol (2:1, v/v)
156 as described previously by Folch *et al.*[9] The fatty acid composition of the extracted
157 lipids was determined by gas chromatography (GC) after the conversion of fatty acyl
158 groups in the lipids to their corresponding methyl esters. Two milliliters of 5%
159 HCl-methanol were added to a sample lipid (20-50 mg) followed by incubation at
160 100°C for 3 h. The HCl-methanol solution was prepared by the dilution of 10%
161 HCl-methanol solution. After cooling the solution, 2 mL of water were added and
162 vortexed, followed by the addition of 2 mL of *n*-hexane. The upper hexane layer
163 containing the methyl esters was recovered and the residual lower layer was further
164 extracted with 2 mL of *n*-hexane. The hexane extracts were combined and washed with
165 water to achieve neutrality. After concentrating the hexane solution under a vacuum, the
166 methyl ester obtained was purified on a silica gel column (silica gel 60; Merck) in an
167 elution with *n*-hexane and a mixture of *n*-hexane-diethyl ether (95:5, v/v). Purified
168 methyl esters were subjected to GC analysis. GC was performed on a Shimadzu
169 GC-14B equipped with a flame-ionization detector and a capillary column [Omegawax
170 320 (30 m x 0.32 mm i.d.); Supelco, Bellefonte, PA]. The injection port and flame
171 ionization detector were set at 250 and 260°C, respectively, and the column temperature
172 was held at 200°C. The carrier gas was helium at a flow rate of 50 kPa. Fatty acid
173 content in the lipid samples was expressed as a weighted percentage of the total fatty
174 acids.
175
176 **Sample collection**
177 Blood samples were taken from caudal vein of the mice without fasting at 0, 7, 14, 21,
178 and 28 days after feeding. Blood glucose was measured using a blood glucose monitor,
179 the Glutest Neo Sensor (Sanwa Kagaku Kenkyusyo Co. Ltd., Aichi, Japan). This sensor
180 is an amperometric sensor with flavin adenine dinucleotide (FAD)-dependent glucose

181 dehydrogenase and $Fe(CN)_6^{3-}$. After feeding with the experimental diets for four weeks,
182 the mice were sacrificed under diethyl ether anesthesia. Blood samples were taken from
183 the caudal vena cava of the mice and each tissue was immediately excised and weighed.
184 The livers were immediately stored in RNA later™ (Sigma Chemical Co., St. Louis,
185 MO) for quantitative real time PCR analysis.
186
187 **Blood lipid analysis**
188 The blood serum analysis of the KK-A^y mice was performed by the Analytical Center of
189 Hakodate Medical Association (Hakodate, Japan). The analysis included the
190 measurement of the following parameters: total cholesterol, neutral lipids (NL), PL,
191 Non-HDL cholesterol, LDL cholesterol, and HDL cholesterol. Blood serum from
192 C57BL/6J mice was extracted with chloroform/methanol (2:1, v/v) according to the
193 method by Folch et al. (1957). The total lipids (TL) extracted were weighed and the
194 serum TAG and cholesterol content were enzymatically measured using commercial kits
195 (Cholesterol E-test and Triglyceride E-test, Wako Pure Chemical Industries Ltd., Osaka,
196 Japan).
197 Some period of fasting is required before glucose tolerance test and needed to obtain
198 stable baseline measurements of blood lipid parameters; however, several recent studies
199 have demonstrated the adverse effect of fasting in rodents.[10,11] During the fasting,
200 especially overnight fasting, they consume much calories and prolonged fasting inhibits
201 insulin-stimulated glucose uptake in humans, but increases the insulin sensitivity in
202 mice. In the present study, we used non-fasting mice for the analysis of blood glucose
203 and serum lipid parameters.
204
205 **Liver Lipid Analysis**
206 TL was extracted from the liver (*ca.* 200 mg) with chloroform/methanol (2:1, v/v).[9] The
207 TL (*ca*. 20 mg) was further separated on a Sep-Pak Silica cartridge (Waters Japan,
208 Tokyo, Japan) by elution with chloroform (50 mL) and methanol (50 mL). The NL and
209 PL fractions were eluted with chloroform and methanol, respectively. Both lipid
210 contents (mg/g liver) in the liver were calculated from the TL level per liver weight. The
211 TAG and cholesterol content in the TL were enzymatically measured using commercial
212 kits as described above.
213 The fatty acid methyl esters from the liver TL were prepared using the method of
214 Prevot and Mordret.[12] Briefly, 1 mL of *n*-hexane and 0.2 mL of 2 N NaOH in methanol
215 were added to an aliquot of total lipid (*ca*. 10 mg), vortexed and incubated at 50°C for
216 30 min. After the incubation, 0.2 mL of 2 N HCl in methanol solution was added to the

217 solution and vortexed. The mixture was separated by centrifugation at 1000 g for 5 min.
218 The upper hexane layer containing fatty acid methyl esters was recovered and subjected
219 to GC. The GC was performed as described above. Each fatty acid level of the liver
220 tissue (1 g) was calculated by comparing the peak ratio to that of the internal standard
221 (17:0) and the total lipid content.
222

223 **Faces analysis**

224 Feces excreted during whole day (24 hr) were collected from a metabolic cage one time
225 (27 day after feeding) for KK-A^y mice and two times (15 or 29 day after feeding) or
226 three times (1, 14, or 27 day after feeding) for C57BL/6J mice. The KK-A^y mice were
227 housed individually; therefore, feces of three days for each animal were individually
228 analyzed. On other hand, C57BL/6J mice of the same experimental group (n=6) were
229 housed in one cage. Thus, feces of 6 animals were analyzed all together. KK-A^y mice
230 were Collection was done and freeze-dried. After freeze-drying and recording the
231 weight, the samples were further dried in a vacuum desiccator and subsequently crushed
232 to pieces in a coffee mill. The lipids were extracted from the dried powder with 10
233 volumes (v/w) of chloroform/methanol (2:1, v/v) and allowed to stand overnight. The
234 solution was filtrated and the filtrates were concentrated under a vacuum using a rotary
235 evaporator. After weighing the feces lipids, a part of the lipids (*ca.* 1 mg) was subjected
236 to the measurement of total cholesterol and total bile acid using the Cholesterol E-Test
237 (Wako Pure Chemical Industries Ltd.) and the Bile Acid Test Wako (Wako Pure
238 Chemical Industries Ltd.), respectively.
239

240 **Quantitative Real-Time PCR**

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

253 (SREBP-2), Mm01282499_m1 (HMG-CoA), Mm00484152 (Cyp7a1),
254 Mm00550438_m1 (CPT1a), Mm00662319_m1 (FAS), Mm00772290_m1 (SCD1),
255 Mm00517086_m1 (Elov2 mRNA), Mm00506717_m1 (Elov5 mRNA),
256 Mm00507605_m1 (Fads1 mRNA), Mm00517221_m1 (Fads2 mRNA), and
257 Mm99999915_g1 (GAPDH mRNA; internal control), respectively.
258

259 **Statistical analysis**
260 Data are presented as the means ± standard error of the mean (SEM) (n=7 or 6). The
261 data were analyzed by a two-way Analysis of variance (ANOVA) using SM and dietary
262 lipids as two variable factors. When no interaction was present between both factors,
263 different groups were compared by Tukey's post hoc analysis. If an interaction was
264 present, one-way ANOVA and t-test were performed between two groups fed the same
265 dietary lipid with or without SM. Differences with $P < 0.05$ were considered
266 significant.
267

268 **Results**
269 **SM separation**
270 Commercial LC-BS contained 51.9 ± 0.69 wt% lipids per dry matter (average ±
271 standard deviation of the mean, n=3). Crude milk SL were concentrated from the
272 LC-BS on the basis of the different distribution of milk lipid class to organic solvents,
273 and then were further separated by selective saponification to remove glycerol-*o*-acyl
274 esters, such as phospholipids. The crude SL (5.51 ± 0.49 g) was recovered from 100 g
275 of LC-BS. Purified SM (1.89 ± 0.53 g) was obtained from the crude SL by silicic acid
276 column chromatography. The SM gave only a single spot and a single peak
277 corresponding to the standard milk SM on TLC and HPLC, respectively. Fatty acid
278 analysis by GC showed that the major fatty acids of SM were long chain saturated fatty
279 acids such as 22:0, 23:0, and 24:0. The GC analysis also showed the major fatty acids of
280 lard (18:1n-9, 16:0, and 18:0), soybean oil (18:2n-6 and 18:1n-9), linseed oil (18:3n-3,
281 18:1n-9, and 18:2n-6), and fish oil (22:6n-3 and 20:5n-3). The fatty acid profile of the
282 lipids extracted from each diet is shown in Table 2. The composition was reflected by
283 the dietary lipids with or without SM (Table 1).
284

285 **Body weights, tissue weights, blood glucose levels, serum and hepatic lipid**
286 **parameters.**
287 There were significant differences in several parameters, namely, water intake and liver
288 weight for KK-A^y mice, and food intake, water intake, liver weight, kidney weight, and

289 small intestine weight for C57BL/6J mice (Table 3). On the other hand, no significant
290 difference was found in body weight, total white adipose tissue (WAT) weight, and
291 blood glucose level in both animal models. There was also no significant difference in
292 the blood glucose levels at 0, 7, 14, 21, and 28 days after feeding.
293 On the other hand, serum total cholesterol, PL, non-HDL cholesterol, and LDL
294 cholesterol levels were affected by the dietary lipids in KK-A^y mice, although little
295 effect was found in the levels of NL and HDL cholesterol (Fig. 1). SM supplementation
296 decreased non-HDL cholesterol levels in soybean oil- and linseed oil-fed mice (Fig. 1
297 D). The same tendency was also observed in total cholesterol (Fig. 1 A) and
298 phospholipids (Fig. 1 C). When the comparison was done between the two groups fed
299 the same dietary lipids with or without SM, the significant decrease in the LDL
300 cholesterol levels was found by SM supplementation in soybean oil- and linseed oil-fed
301 mice (Fig. 1 E). A similar dietary effect was found in the liver lipid content (Figure 2).
302 The reducing effect of SM supplementation was found on the TL (Fig. 2 A), NL (Fig. 2
303 B), TAG (Fig. 2 D), and cholesterol (Fig. 2 E) levels in the liver of KK-A^y mice, though
304 there were no significant differences. The soybean and linseed oil fed groups had
305 significantly lower levels of TL and NL than the lard group. Overall, Fig. 1 and 2
306 indicate the combined effect of SM supplementation with dietary fat containing
307 polyunsaturated fatty acids (PUFA), such as linoleic acid (18:2n-6, LA) and α-linolenic
308 acid (18:3n-3, ALA) (Table 2) on the reduction of serum and liver lipids. In contrast to
309 the KK-A^y mice, no decrease in serum and liver lipids was observed in the C57BL/6J
310 mice (Fig. 3 and 4).
311

312 **Fecal lipids.**

313 The fecal concentrations of TL, cholesterol, and PL were also affected by dietary lipids
314 (Fig. 5 and 6). In the KK-A^y mice, the fecal TL content was the lowest in the linseed
315 oil-fed group; however, the level (88.7+41.2 mg/g feces) significantly increased to
316 221.7±27.6 mg/g feces following SM supplementation (Fig. 5 A). The excretion of total
317 lipids in the feces was promoted by SM supplementation in the lard- and soybean-fed
318 groups. Fecal cholesterol levels were also increased by SM supplementation and
319 significant difference between with or without SM diets were found in the lard- and
320 linseed oil-fed groups (Fig. 5 B). Bile acid was also significantly increased by SM
321 supplementation in lard-fed group, while there was no significant effect of SM
322 supplementation in other two groups (Fig. 5 C). The increase in fecal TL was also found
323 in C57BL/6J mice (Fig. 6). Although statistical analyses could not be performed
324 because the experimental groups with C57BL/6J mice were housed in the same cage,

325 the result in Fig. 6 strongly suggests the greater excretion of TL in the feces following
326 SM supplementation in C57BL/6J mice. To confirm the promotion of TL secretion into
327 the feces in C57BL/6J mice by SM supplementation, separate animal experiments have
328 been performed using dietary lipids containing 230 g/kg lard and 70 g/kg soybean oil or
329 60 g/kg soybean oil + 10 g/kg SM. When both diets (Table 1) were given to wild-type
330 C57BL/6J mice (male, four weeks old, n=7) for 29 days, a significant increase in the TL
331 in the feces following SM supplementation was observed 15 and 29 days after feeding
332 (Fig. 7).
333

334 **Fatty acid levels of liver lipids and gene expression**
335 Lard diets contained higher levels of saturated and monounsaturated fatty acids, such as
336 16:0 and 18:1n-9, than the other dietary groups (Table 2). Thus, high levels of 16:0 and
337 18:1n-9 were found in the liver lipids of lard-fed KK-A^y mice (Table 4). The
338 characteristic fatty acid compositions of the soybean oil and linseed oil groups were
339 high levels of LA and ALA, respectively (Table 2). Both PUFA were also found at
340 relatively high concentrations in the liver lipids of soybean oil- and linseed oil-fed
341 KK-A^y mice, respectively (Table 4). On the other hand, SM supplementation
342 significantly reduced the total fatty acid content (mg/1 g liver) of the lard-fed mice. The
343 same tendency was found in the total fatty acid contents of the soybean oil- and linseed
344 oil-fed mice. The decrease in the total fatty acids, presented in Table 4, was consistent
345 with the result in Fig. 2 showing the reducing effect of the SM supplementation on liver
346 TL (Fig. 2 A) and NL (Fig. 2 B). Table 4 also shows the decrease in saturated and
347 monounsaturated fatty acids, LA, and ALA by SM supplementation; however,
348 arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA),
349 docosapentaenoic acid (22:5n-3, DPA), and docosahexaenoic acid (22:6n-3, DHA)
350 increased in the SM supplemented soybean oil- and linseed oil-fed groups.
351 To determine the effect of dietary lipids on liver lipid metabolism in KK-A^y mice,
352 the related gene expressions were analyzed by real-time PCR. The analysis showed no
353 significant effect of dietary lipids on the gene expression related to cholesterol
354 metabolism (SREBP-2, HMG-CoA, and Cyp7a1) (Fig. 8) and on the expression of FAS
355 and CPT1a. On the other hand, a difference was found in SCD1 gene expression, with a
356 conversion from 16:0 and 18:0 to 16:1n-7 and 18:1n-9. Table 4 presents the
357 significantly higher levels of 18:1n-9/18:0 in the lard-fed group compared to the
358 soybean and linseed oil-fed groups. On the other hand, a significant decrease in
359 16:1n-7/16:0 was observed due to SM supplementation in the soybean oil-fed group.
360 The decreasing trend in 18:1n-9/18:0 was also observed due to SM supplementation in

361 soybean oil- and linseed oil-fed groups. The change in the ratio of monoenoic fatty
362 acid/saturated fatty acid was consistent with the dietary up- and down-regulation of
363 SCD1 mRNA presented in Fig. 9. LA and ALA are converted to ARA and DHA,
364 respectively, through a series of desaturation and chain elongation processes including
365 Elov2, Elov5, Fads1, and Fads2. The different diet feeding resulted in the significant
366 changes in the expression of Elov2, Elov5, and Fads2 (Fig. 9), while no significant
367 difference was found in the expression of Fads1.
368 On the other hand, no decrease in the liver total fatty acids was observed in
369 C57BL/6J mice following SM supplementation (Table 5). The fatty acid composition of
370 the liver lipids was well reflected by the dietary lipids to show the high level of ALA
371 and DHA in the linseed oil- and fish oil-fed group, respectively; however, no specific
372 effect of SM supplementation was found on the fatty acid composition including
373 18:1n-9/18:0 and 16:1n-7/16:0 ratio. In addition, there was no significant difference in
374 gene expression related to lipid metabolism with or without SM supplementation.
375
376 **Discussion**
377 SM is an essential biological component with important roles, such as cell membrane
378 formation, lipid microdomains functionality, and signal transduction.[14-15] On the other
379 hand, SM is a dietary component with an average consumption per capita in the
380 Western diet of ~200-400 mg/day.[16,17] Studies have examined the effects of dietary SM
381 and have found reductions of the liver and plasma lipid levels.[18] In the present study, we
382 also found that dietary SM could reduce the liver and plasma lipid levels of
383 obese/diabetic KK-A^y mice (Fig. 1, 2, and Table 4). This effect was mainly dependent
384 on the increase in fecal TL and cholesterol observed in the mice fed SM (Fig. 5).
385 Inhibition of the intestinal lipid absorption by SM has also been reported as the probable
386 mechanism for the lowering effect of SM on the liver and/or plasma lipid levels.[19-22]
387 This effect of SM is due to its physical property of being relatively resistant to
388 solubilization into bile salt micelles. The low solubility of SM induces its incomplete
389 hydrolysis in the upper segment of the intestine, where much of lipid hydrolysis
390 occurs.[23] The slow and incomplete hydrolysis of SM may allow for interactions between
391 intact SM and other lipids in the luminal environment, lowering the rates of hydrolysis,
392 micellar solubilization and the transfer of lipids from mixed micelles to the enterocyte.
393 [21]
394 Although the inhibition of the intestinal lipid absorption by dietary SM has been
395 made clear using physico-chemical model,[24] cellular
396 model,[25,26] ^{3}H-dihydrosphingosine-labeled SM,[17] and lymph cannulation method,[21]

397 research of the effect of dietary SM in animal models has been limited. Duivenvoorden
398 *et al*.[18] reported the lowering effect of SM on plasma lipid levels of hyperlipidemic
399 *APOE*3*Leiden mice fed a Western-type diet. The same effect has been reported in
400 obese Zucker rats.[22] The present study also confirmed the reduction of serum and liver
401 lipid levels in obese/diabetic mice through the promotion of intestinal lipid secretion by
402 SM. Dietary SM also promoted fecal lipids in wild-type C57BL/6J mice (Fig. 6 and 7);
403 however, no decrease in serum and liver lipids was observed in the wild-type mice (Fig.
404 3 and 4). This may suggest the resistance of normal conditions to changes in the lipid
405 profiles of biological systems. Our present finding on the different effect of SM on
406 obese/diabetic and wild-type model mice suggests the possibility of effectiveness of SM
407 on human subjects with metabolic abnormalities. However, cholesterol metabolism of
408 mice is different from that of human. A human study reported no significant changes in
409 the plasma lipid profile after the consumption of SM.[23,27,28] Ramprasath *et al*.[23]
410 demonstrated some limitations of human studies: e.g., sample size, the SM containing
411 diet formulation, and the dose level of SM. In addition, these human studies have been
412 conducted with only healthy subjects; therefore, more studies to determine whether SM
413 affects cholesterol absorption and plasma lipids in hyperlipidemic subjects are needed.
414 Recently, much attention has been paid to the health beneficial effects of milk
415 SM.[29-31] When milk SM was given to C57BL/6J mice fed high fat diet, significantly
416 reduction was found in body weight, serum cholesterol and hepatic triglycerides
417 levels.[29] On the contrary, the same level of egg SM supplementation increased the
418 serum cholesterol, triglycerides, phospholipids, and hepatic triglycerides.[29] Lecomte *et*
419 *al*.[31] reported that the supplementation soybean polar lipids to C57BL/6J mice fed high
420 fat diet significantly increased the hepatic lipid levels, while there was little effect of
421 milk polar lipids on the hepatic lipid levels. It is apparent that supplementation of SM
422 including egg and soybean SM could inhibit lipid absorption in animal
423 models.[19,21,24-26,32] To compensate for the reduction of absorbed lipids, hepatic de novo
424 lipogenesis would be up-regulated. Norris *et al*.[29] found that milk SM feeding
425 significantly increased hepatic HMG-CoA and SREBP2 gene expressions of C57BL/6J
426 mice fed high fat diet. On the other hand, milk SM supplementation significantly
427 decreased serum total cholesterol and hepatic TAG, although the reverse effect was
428 found in egg SM supplementation.[21,29] Moreover, egg SM feeding significantly
429 increased SCD1 gene expressions, while no increase in SCD1 was found in the mice fed
430 milk SM.[29] In the present study, milk SM supplementation decreased SCD1 of KK-A^y
431 mice (Fig. 9A), while the increase in HMG-CoA and SREBP2 gene expressions was
432 observed (Fig. 8 A and B).

433 ALA is an essential fatty acid that must be consumed through diet. There have been
434 many epidemiological and clinical studies on the cardiovascular-protective effects of
435 ALA.[33] LA is a precursor of EPA and DHA. Both n-3 EPA and DHA have been
436 regarded as active forms of ALA in biological systems. EPA and DHA have been shown
437 to cause significant biochemical and physiological changes in the body that often have a
438 positive influence on human nutrition and health. EPA and DHA can reduce serum and
439 liver lipid levels due to the regulation of lipid metabolism.[33,34] Because linseed oil is
440 rich in ALA (Table 2), a combination effect was found in linseed oil + SM
441 supplementation in KK-A^y mice (Fig. 1 and 2). Compared with lard alone, linseed oil +
442 SM supplementation could significantly reduce serum total cholesterol, non-HDL
443 cholesterol, and LDL cholesterol (Fig. 1) and liver TL, NL, and cholesterol (Fig. 2).
444 SM supplementation reduced the intestinal lipid absorption in KK-A^y mice (Fig. 5).
445 This might induce the decreasing tendency of hepatic TL (Fig. 2). Hepatic total fatty
446 acids analyses also confirmed this effect of SM. Table 4 presents the significant
447 decrease in hepatic total fatty acids of the lard-fed group with SM supplementation
448 compared to mice without SM. The decrease in total fatty acids by SM supplementation
449 was also found in the soybean oil- and linseed oil-fed mice, but the difference was not
450 significant. In the soybean oil- and linseed oil-fed groups, SM supplementation
451 increased long-chain PUFA such as ARA, EPA, DPA, and DHA (Table 4). The increase
452 in EPA, DPA, and DHA, the active n-3 PUFA forms of ALA, may be related to the
453 reduction of serum and liver lipid levels in the soybean oil- and linseed oil-fed KK-A^y
454 mice supplemented with SM (Fig. 1 and 2). The increase in n-3 PUFA might be induced
455 by the up-regulation of ALA bioconversion to EPA, DPA, and DHA; however, no
456 change was observed in the related gene expressions, namely, Δ^6-desaturase (Fads2),
457 elongase-5 (Elov5), Δ^5-desaturase (Fads1), and elongase-2 (Elov2) (Fig. 9). Another
458 notable effect of SM supplementation was a decrease in liver 18:1n-9/18:0 and
459 16:1n-7/16:0 ratios in the soybean oil- and linseed oil-fed KK-A^y mice (Table 4). In our
460 previous study (Watanabe *et al*. 2011), a significant decrease in 18:1n-9 was found in
461 the liver lipids of the KK-A^y mice fed milk SL. The levels of 16:1n-7 and 18:1n-7 were
462 also reduced by the SL feeding. Thus, the down-regulation of SCD1 by milk SL has
463 been suggested. The present study demonstrates the reduction of the SCD1 gene
464 expression by SM supplementation (Fig. 9A).
465 The different activity of SM on wild-type and obese/diabetic mice suggests the
466 possibility that SM may be useful for the improvement of hyperlipidemia in subjects
467 with metabolic disorders. The major mechanism for this effect will be the promotion of
468 intestinal lipid secretion. On the other hand, further studies may be needed to investigate

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

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

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

491

492 **References**

493 1. V. L. Spitsberg, Inivited review: bovine milk fat globule membrane as a potential
494 nutraceuticals, *J. Dairy Sci.*, 2005, **88**, 2289-2294.
495 2. G. Contarini and M. Povolo, Phospholipids in milk fat: composition, biological and
496 technological significance, and analytical strategies, *Int. J. Mol. Sci.* 2013, **14**,
497 2808-2831.
498 3. E. Wat, S. Tandy, E. Kapera,A. Kamili, R. W. S. Chung, A. Brown, M. Rowney and
499 J. S. Cohn, Dietary phospholipid-rich dairy milk extract reduces hepatomegaly,
500 hepatic steatosis and hyperlipidemia in mice fed a high-fat diet, *Atherosclerosis*
501 2009, **205**, 144-150.
502 4. A. Kamili, E. Wat, R. W. S. Chung, S. Tandy, J. M. Weir, P. J. Meikle and J. S.
503 Cohn, Hepatic accumulation of intestinal cholesterol is decreased and fecal
504 cholesterol excretion is increased in mice fed a high-fat diet supplemented with milk

505 phospholipids, *Nutr. Metab.*, 2010, **7**, 90-101.
506 5. S. Watanabe, T. Takahashi, L. Tanaka, Y. Haruta, M. Shiota, M. Hosokawa and K.
507 Miyashita, The effect of milk polar lipids separated from butter serum on the lipid
508 levels in the liver and the plasma of obese-model mouse (KK-Ay), *J. Functional*
509 *Foods* 2011, **3**, 313-320.
510 6. S. Keller, A. Malarski, C. Reuther, R. Kertscher, M. Kiehntopf and G. Jahreis, Milk
511 phospholipid and plant sterol-dependent modulation of plasma lipids in healthy
512 volunteers, *Eur. J. Nutr.*, 2013, **52**, 1169-1179.
513 7. J. C. Astaire, R. Ward, J. B. German and R. Jiménez-Flores, Concentration of polar
514 MFGM lipids from buttermilk by microfiltration and supercritical fluid extraction, *J.*
515 *Dairy Sci.*, 2003, **86**, 2297-2307.
516 8. L. Stillway and S. J. Harmon, A procedure for detecting phosphonolipids on
517 chromatograrns, *J. Lipid Res.*, 1980, **21**, 1141-1143.
518 9. J. Folch, M. Lees and G. H. S. Stanley, A simple method for the isolation and
519 purification of total lipides from animal tissues, *J. Biol. Chem.* 1957, **226**, 497-509.
520 10. E. Razmpoosh, M. Javadi, H.-S. Ejtahed and P. Mirmiran, Probiotics as beneficial
521 agents in the management of diabetes mellitus: a systematic review, *Diabetes Metab.*
522 *Res. Rev.* 2016, **32**, 143-168.
523 11. J. E. Bowe, Z. J. Franklin, A. C. Hauge-Evans, A. J. King, S. J. Persaud and P. M.
524 Jones, Assessing glucose homeostasis in rodent models, *J. Endocrinol.*, 2014, **222**,
525 G13-25.
526 12. A. F. Prevot and F. X. Mordret, Utilisation des colonnes capillaries de verre pour
527 l'analyse des corps gras par chromotographie en phase gazeuse, *Rev. Fse. Corps.*
528 *Grasl.*, 1976, **23**, 409-423.
529 13. R. -D. Duan and A. Nilsson, Metabolism of sphingolipids in the gut and its relation
530 to inflammation and cancer development, *Prog. Lipid Res.*, 2009, **48**, 62-72.
531 14. P. J. Quinn, Lipid matrix model of membrane raft structure, *Prog. Lipid Res.*, 2010,
532 **49**, 390-406.
533 15. B. M. Castro, M. Prieto L. C. Silva, A simple sphingolipid with unique biophysical
534 properties, *Prog. Lipid Res.*, 2014, **54**, 53-67.
535 16. H. Vesper, E. M. Schmelz, M. N. Nikolova-Karakashian, D. L. Dillehay, D. V.
536 Lynch and A. H. Merrill, Sphingolipids in food and the emerging importance of
537 sphingolipids to nutrition, *J. Nutr.*, 1999, **129**, 1239-1250.
538 17. M. Lecomte, C. Bourlieu, E. Meugnier, A. Penhoat, D. Cheillan, G. Pineau, E.
539 Loizon, M. Trauchesse, M. Claude, O. Ménard, A. Géloën, F. Laugerette and M. -C.
540 Michalski, Milk polar l ipids affect in vitro digestive lipolysis and postprandial lipid

541 metabolism in mice, *J. Nutr.*, 2015, **145**, 1770-1777.
542 18. I. Duivenvoorden, P. J. Voshol, P. C. N. Rensen, W. van Duyvenvoorde, J. A.
543 Romijn, J. J. Emeis, L. M. Havekes and W. F. Nieuwenhuizen, Dietary
544 sphingolipids lower plasma cholesterol and triacylglycerol and prevent liver
545 steatosis in APOE*3Leiden mice, *Am. J. Clin. Nutr.*, 2006, **84**, 312-321.
546 19. L. Nyberg, R. D. Duan and A. Nilsson, A mutual inhibitory effect on absorption of
547 sphingomyelin and cholesterol, *J. Nutr. Biochem.*, 2000, **11**, 244-249.
548 20. E. R. M. Eckhardt, D. Q. –H. Wang, J. M. Donovan and M. C. Carey, Dietary
549 shingomyelin suppresses intestinal cholesterol absorption by decreasing
550 thermodynamic activity of cholesterol monomers, *Gastroenterology*, 2002, **122**,
551 948-956.
552 21. S. K. Noh and S. I. Koo, Egg sphingomyelin lowers the lymphatic absorption of
553 cholesterol and α-tocopherol in rats, *J. Nutr.*, 2003, **133**, 3571-3576.
554 22. K. Yunoki, M. Renaguli, M. Kinoshita, H. Matsuyama, S. Mawatari, T. Fujino, Y.
555 Kodama, M. Sugiyama and M. Ohnishi, Dietary sphingolipids ameliorate disorders
556 of lipid metabolism in Zucker fatty rats, *J. Agric. Food Chem.*, 2010, **58**, 7030-7035.
557 23. V. R. Ramprasath, P. J. H. Jones, D. D. Buckley, L. A. Woollett and J. E. Heubi,
558 Effect of dietary sphingomyelin on absorption and fractional synthetic rate of
559 cholesterol and serum lipid profile in humans, *Lipids Health Dis.*, 2013, **12**, 125.
560 24. K. L. van Erpecum and M. C. Carey, Influence of bile salts on molecular
561 interactions between sphingomyelin and cholesterol: relevance to bile formation and
562 stability, *Biochim. Biophys. Acta*, 1997, **1345**, 269-282.
563 25. H. Chen, E. Born, S. N. Mathur, F. C. Jr. Johlin and F. J. Field, Sphingomyelin
564 content of intestinal cell membranes regulates cholesterol absorption, *Biochem. J.*,
565 1992, **286**, 771-777.
566 26. A. Nilsson and R. D. Duan, Absorption and lipoprotein transport of sphingomyelin,
567 *J. Lipid Res.*, 2006, **47**, 154-171.
568 27. L. Ohlsson, H. Burling and A. Nilsson, Long term effects on human plasma
569 lipoproteins of a formulation enriched in butter milk polar lipid, *Lipids Health Dis.*,
570 2009, **8**, 44.
571 28. L. Ohlsson, H. Burling, R. D. Duan and A. Nilsson, Effects of a sphingolipid
572 enriched dairy formulation on postprandial lipid concentrations, *Eur. J. Clin. Nutr.*,
573 2010, **64**, 1344-1349.
574 29. G. H. Norris, C. Jiang, J. Ryan, C. M. Porter and C. N. Blesso. Milk sphingomyelin
575 improves lipid metabolism and alters gut microbiota in high fat diet-fed mice, *J.*
576 *Nutr. Biochem.*, 2016, **30**, 93-101.

577 30. A. Baars, A. Oosting, E. Engels, D. Kegler, A. Kodde, L. Schipper, H. J. Verkade
578 and E. M. van der Beek. Milk fat globule membrane coating of large lipid droplets
579 in the diet of young mice prevents body fat accumulation in adulthood, Br. J. Nutr.,
580 2016, **115**, 1930-1937
581 31. M. Lecomte, L. Couëdelo, E. Meugnier, P. Plaisancié, M. Létisse, B. Benoit, L.
582 Gabert, A. Penhoat, A. Durand, G. Pineau, F. Joffre, A. Géloën, C. Vaysse, F.
583 Laugerette and M. -C. Michalski, Dietary emulsifiers from milk and soybean
584 differently impact adiposity and inflammation in association with modulation of
585 colonic goblet cells in high-fat fed mice, *Mol. Nutr. Food Res.*, 2016, **60**, 609-620.
586 32. R. W. S. Chung, A. Kamili, S. Tandy, J. M. Weir, R. Gaire, G. Wong, P. J. Meikle,
587 J. S. Cohn and K. -A. Rye, Dietary sphingomyelin lowers hepatic lipid levels and
588 inhibits intestinal cholesterol absorption in high-fat-fed mice, *PLoS One*, 2013, **8**,
589 e55949.
590 33. K. -B. Kim, Y. A. Nam, H. S. Kim, A. W. Hayes and B. -M. Lee, α-Linolenic acid:
591 nutraceutical, pharmacological and toxicological evaluation, *Food Chem. Toxicol.*,
592 2014, **70**, 163-178.
593 34. T. A. Mori, Dietary n-3 PUFA and CVD: a review of the evidence, *Proc. Nutr. Soc.*,
594 2014, **73**, 57-64.
595 35. M. T. Flowers and J. M. Ntambi, Role of stearoyl-coenzyme A
596 desaturase in regulating lipid metabolism, *Curr. Op. Lipid.*, 2008, **19**, 248-256.
597 36. M. T. Flowers and J. M. Ntambi, Stearoyl-CoA desaturase and its relation to
598 high-carbohydrate diets and obesity, *Biochim. Biophys. Acta*, 2009, **1791**, 85-91.
599 37. C. M. Paton and J. M. Ntambi, Biochemical and physiological
600 function of stearoyl-CoA desaturase, *Am. J. Physiol.*, 2008, **297**, E28-E37.
601 38. L. Nyberg, R. D. Duan, J. Axelson and A. Nilsson, Identification of an alkaline
602 sphingomyelinase activity in human bile, *Biochim. Biophys. Acta*, 1996, **1300**,
603 42-48.
604
605 Figure legends
606
607 **Figure 1.** The effects of dietary lipids on serum lipid parameters of KK-A^y mice. (A),
608 Total cholesterol; (B), NL; (C), PL; (D), non-HDL cholesterol; (E), LDL cholesterol;
609 (F), HDL cholesterol. Values represent the means ± SEM of seven mice per group. A
610 two-way ANOVA analysis showed that serum lipid parameters except for LDL
611 cholesterol were not affected by the interaction of dietary lipids and SM feeding.
612 Therefore, the significance was compared by Tukey's post hoc analysis except for LDL

613 cholesterol. Different letters (a, b, c) show significant differences at $P < 0.05$. The
614 comparison of LDL cholesterol was done with one way ANOVA and t-test on two
615 groups fed the same dietary lipid with or without SM ($^{\#}P$ <0.05 vs without SM) .
616

617 **Figure 2.** The effects of dietary lipids on liver lipid levels of KK-A^y mice. (A), TL;
618 (B), NL; (C), PL; (D), TAG; (E), cholesterol. Values represent the means ± SEM of
619 seven mice per group. A two-way ANOVA analysis showed that all lipid parameters
620 were not affected by the interaction of dietary lipids and SM feeding. Therefore, the
621 significance was compared by Tukey's post hoc analysis. Different letters (a, b, c) show
622 significant differences at $P < 0.05$.
623

624 **Figure 3.** The effects of dietary lipids on serum lipid levels of C57BL/6J mice. (A),
625 TL; (B), TAG; (C), cholesterol. Values represent the means ± SEM of six mice per
626 group. A two-way ANOVA analysis showed that all lipid parameters were not affected
627 by the interaction of dietary lipids and SM feeding. The analysis also showed no
628 significant difference between the groups (P <0.05).
629

630 **Figure 4.** The effects of dietary lipids on liver lipid levels of C57BL/6J mice. (A), TL;
631 (B), NL; (C), PL; (D), TAG; (E), cholesterol. Values represent the means ± SEM of six
632 mice per group. A two-way ANOVA analysis showed that all lipid parameters were not
633 affected by the interaction of dietary lipids and SM feeding. The analysis also showed
634 no significant difference between the groups (P <0.05).
635

636 **Figure 5.** The effects of SM supplementation on fecal lipid levels of KK-A^y mice fed
637 lard, soybean oil, and linseed oil. (A), TL; (B), cholesterol; (C), bile acid. Values
638 represent the means ± SEM of seven mice per group. A two-way ANOVA analysis
639 showed that all lipid parameters were significantly ($P < 0.05$) affected by the interaction
640 of dietary lipids and SM feeding. Therefore, the comparison of lipid parameters were
641 done with one way ANOVA and t-test on two groups fed the same dietary lipid with or
642 without SM ($^{\#}P$ <0.05 vs without SM) .
643

644 **Figure 6.** The effects of SM supplementation on fecal TL levels of C57BL/6J mice
645 fed linseed and fish oil after 1 day (A), 14 days (B), 27 days (C) of feeding. Values
646 represent the means of six mice per group.
647

648 **Figure 7.** The effects of SM supplementation on fecal TL levels of C57BL/6J mice
649 fed soybean oil after 15 days (A) and 29 days (B) of feeding. Values represent the
650 means ± SEM of six mice per group. The comparison was done with one way ANOVA
651 and t-test on two groups with or without SM ($^{\#}P$ <0.05 vs without SM) .
652
653 **Figure 8.** The gene expressions of the liver associated with cholesterol metabolism in
654 KK-A^y mice fed different dietary lipids. Values represent the means ± SEM of seven
655 mice per group. A two-way ANOVA analysis showed that all lipid parameters were not
656 affected by the interaction of dietary lipids and SM feeding. The analysis also showed
657 no significant difference between the groups (P <0.05).
658
659 **Figure 9.** The gene expressions of the liver associated with the bioconversion of ALA
660 to DHA in KK-A^y mice fed different dietary lipids. Values represent the means ± SEM
661 of seven mice per group. A two-way ANOVA analysis showed that all lipid parameters
662 were not affected by the interaction of dietary lipids and SM feeding. Therefore, the
663 significance was compared by Tukey's post hoc analysis. Different letters (a, b, c) show
664 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

Fatty acid	KK-A^y mice						C57BL/6J mice			
	Lard	Lard +SM	Soybean oil	Soybean oil + SM	Linseed oil	Linseed oil + SM	Linseed oil	Linseed oil + SM	Fish oil	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 $P < 0.05$.

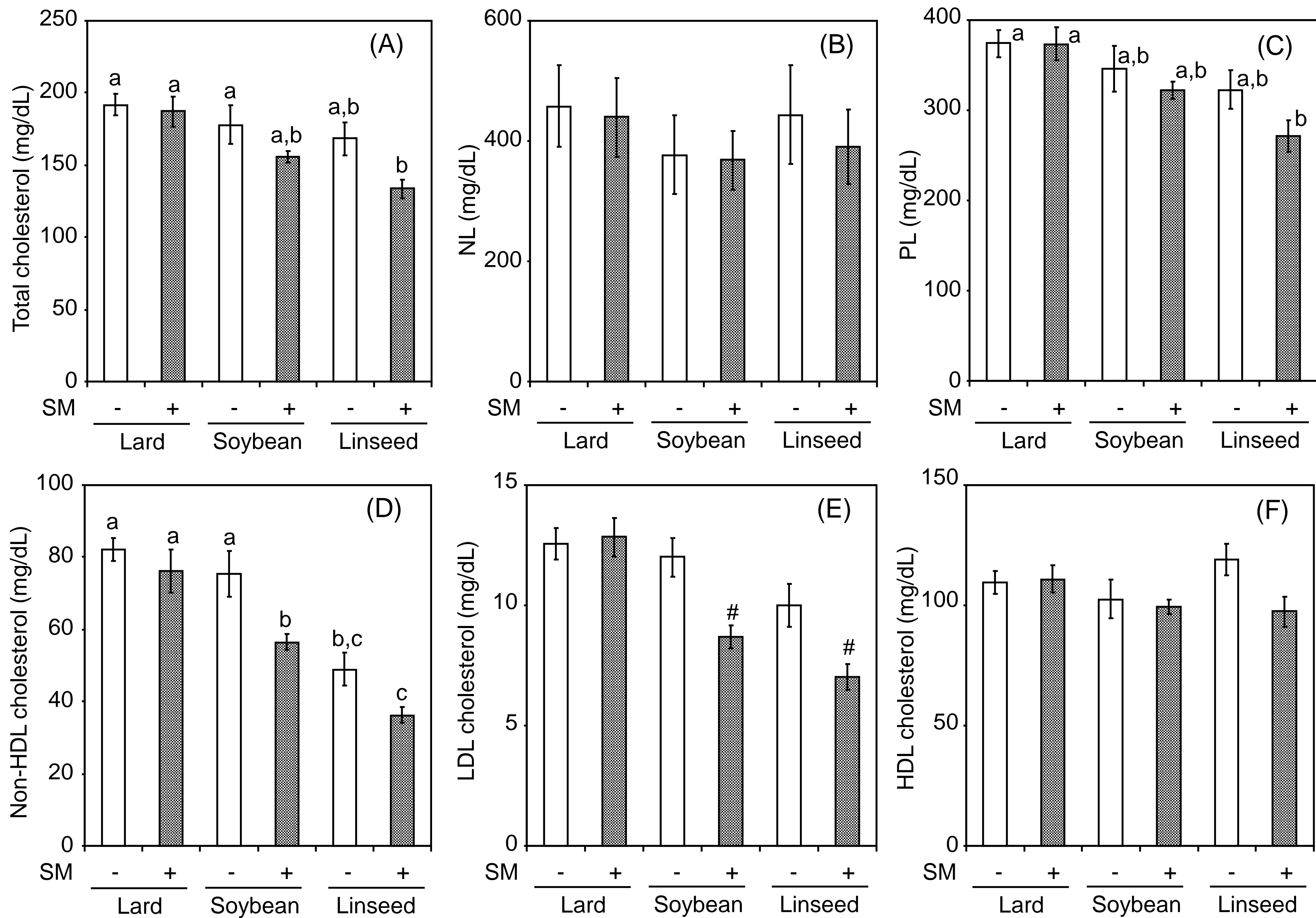
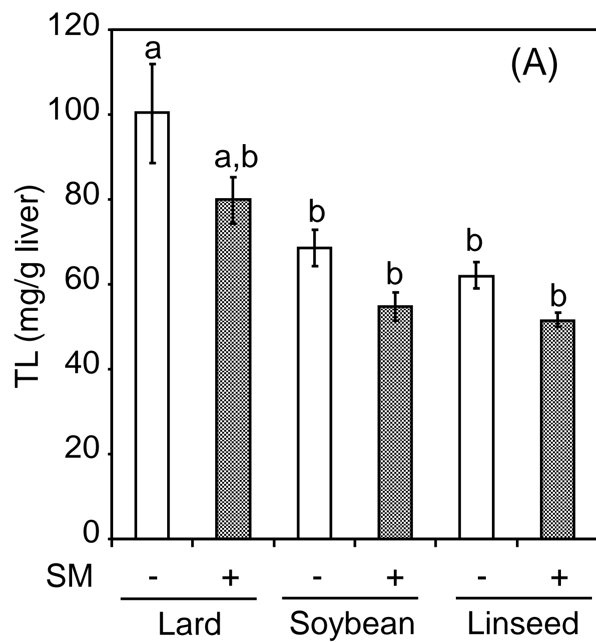
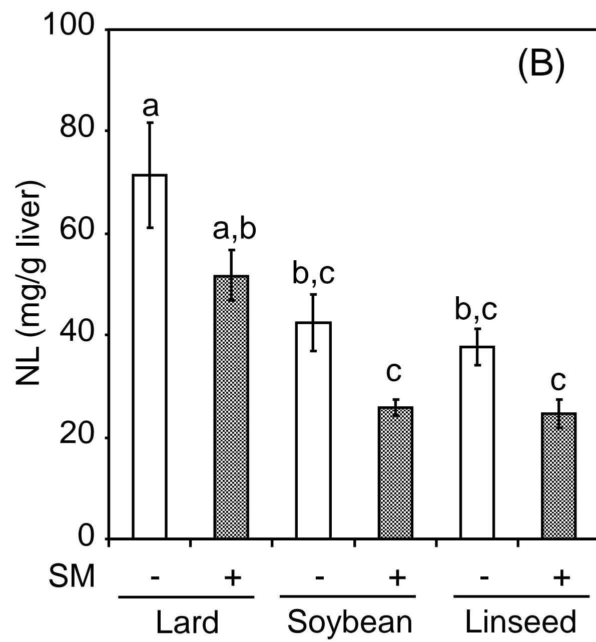
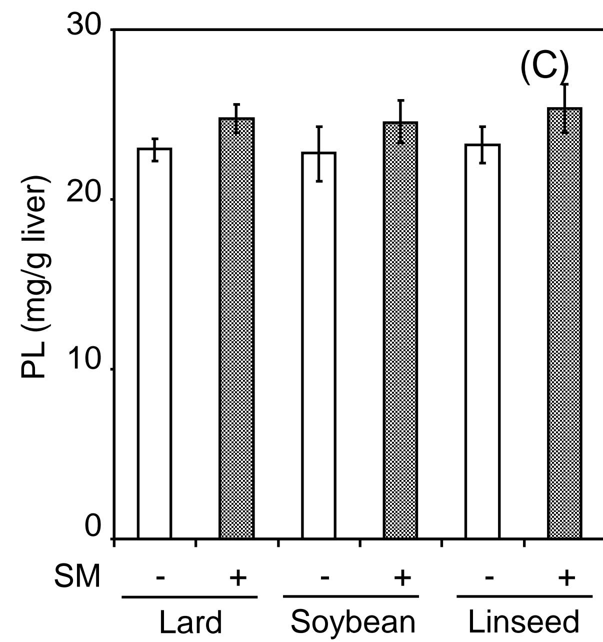
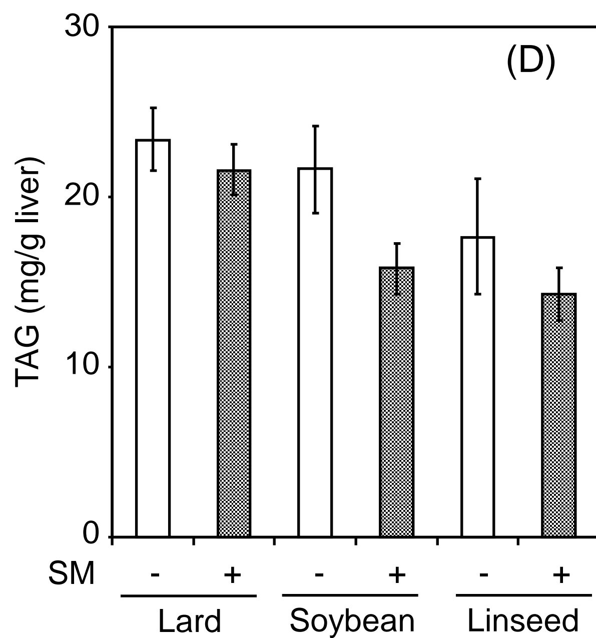


Fig. 1









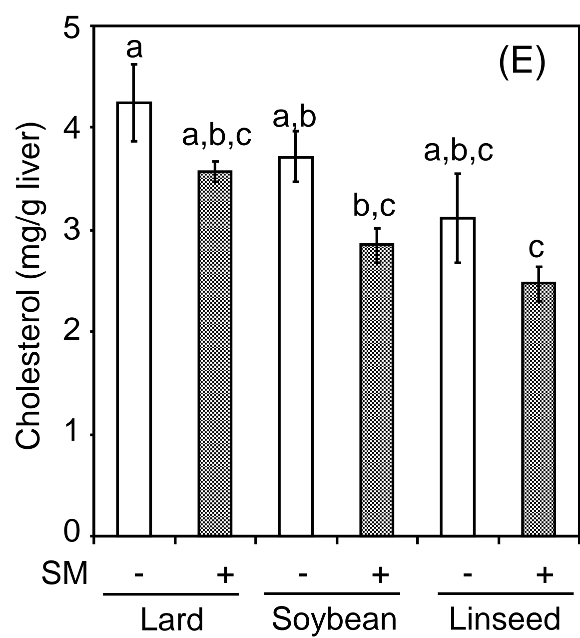


Fig. 2

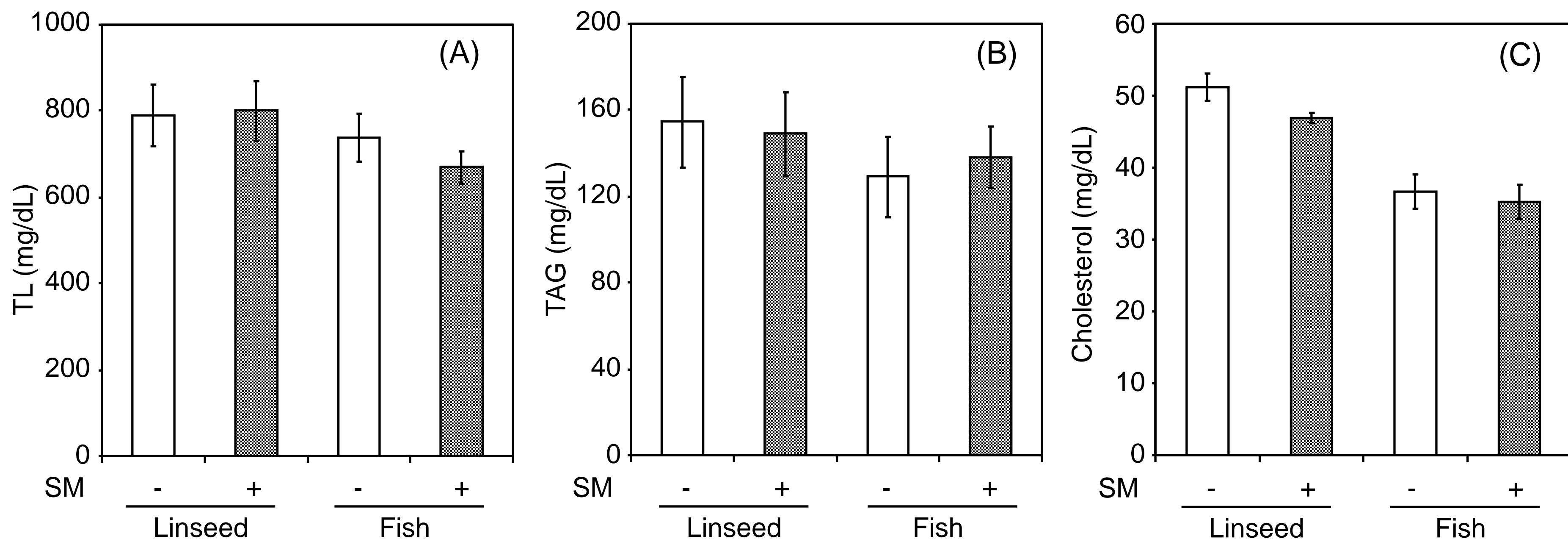


Fig. 3

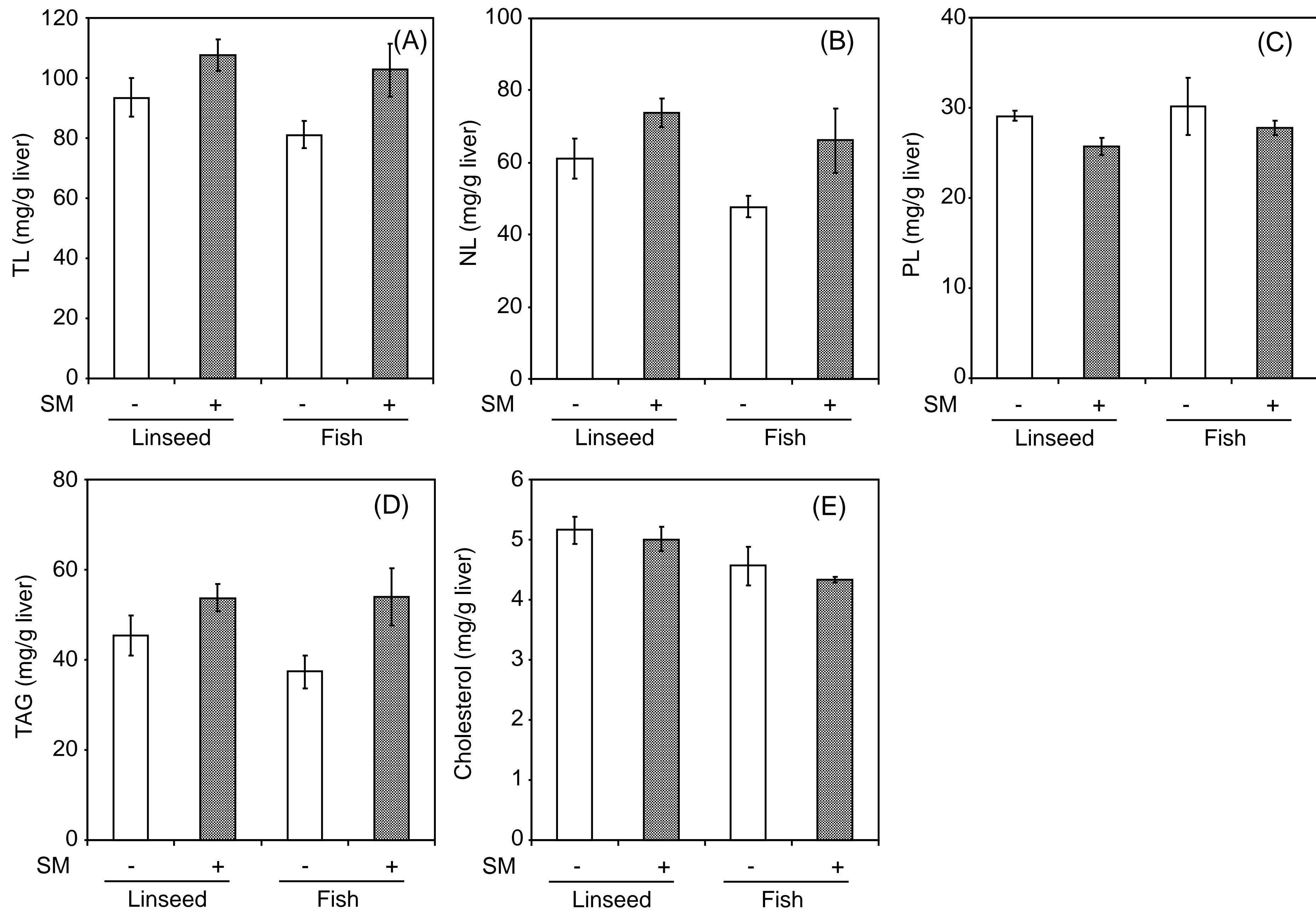
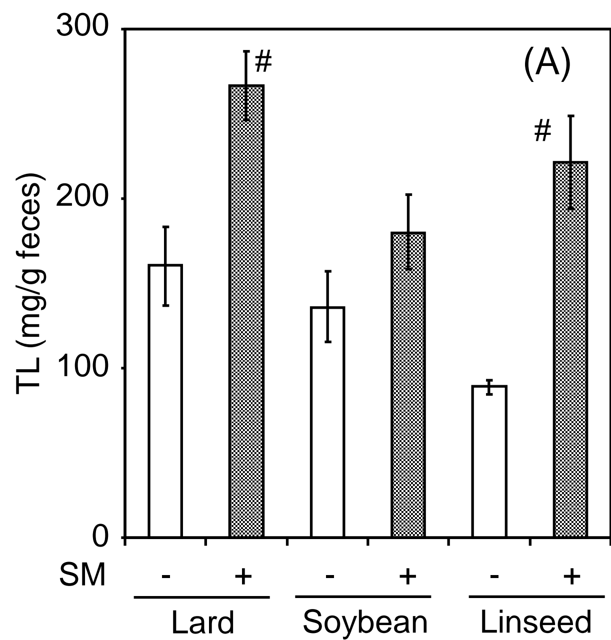
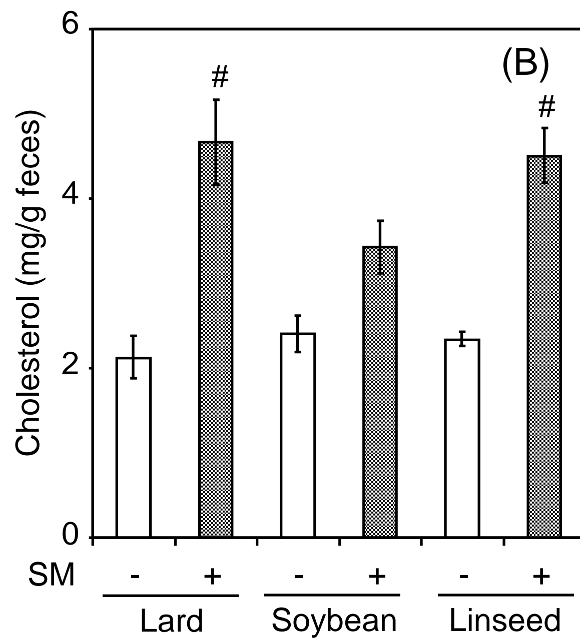


Fig. 4





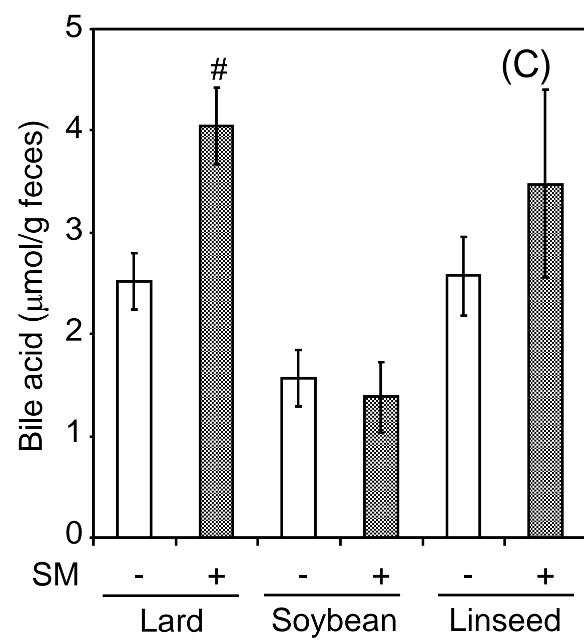


Fig. 5

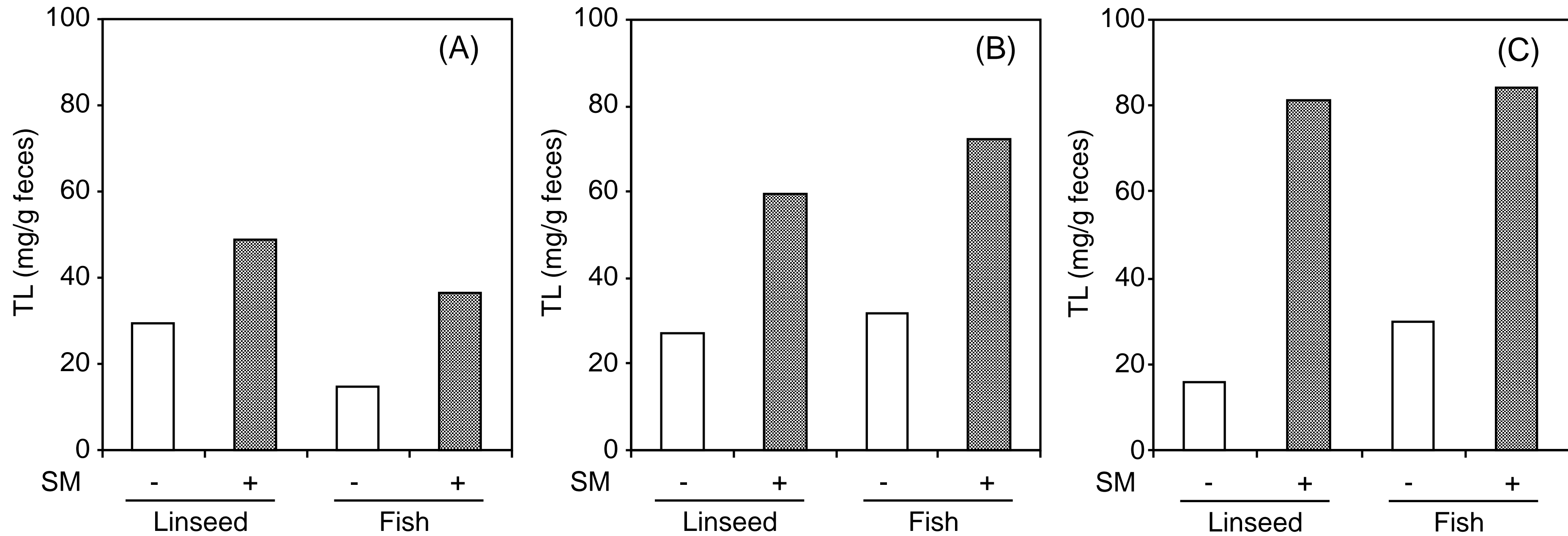
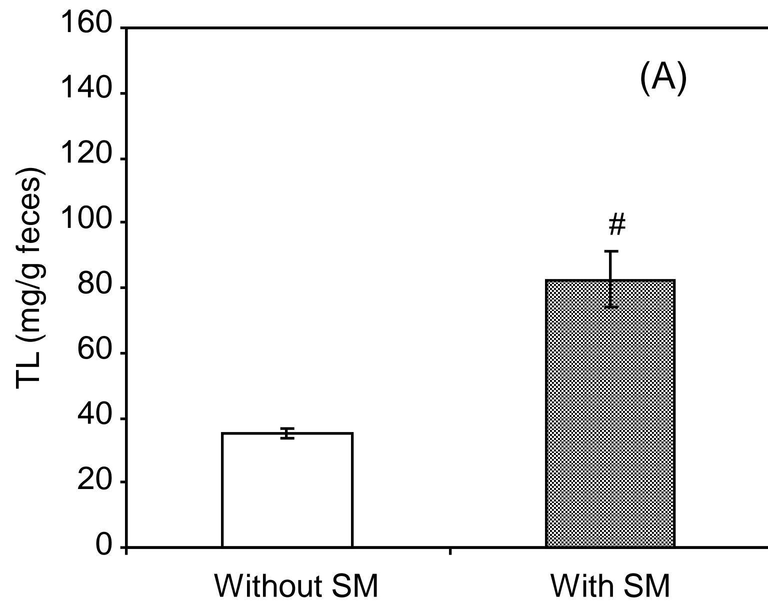


Fig. 6



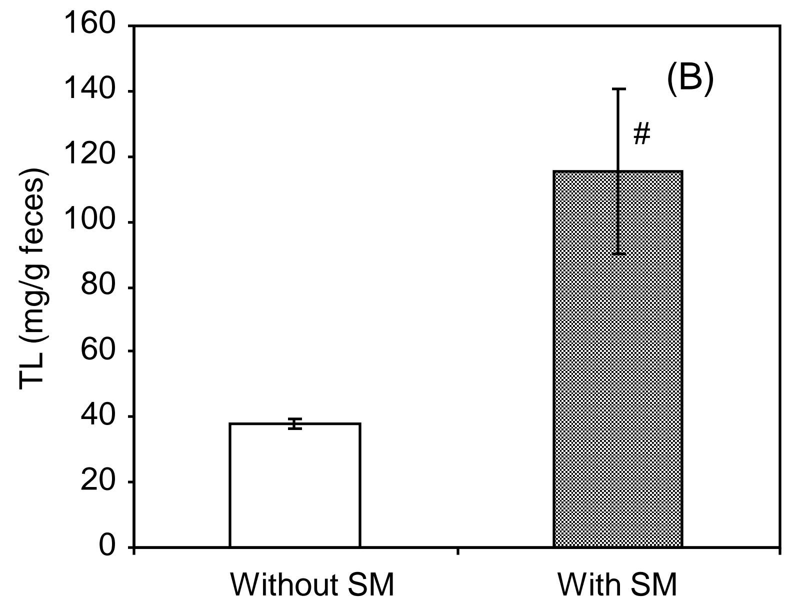
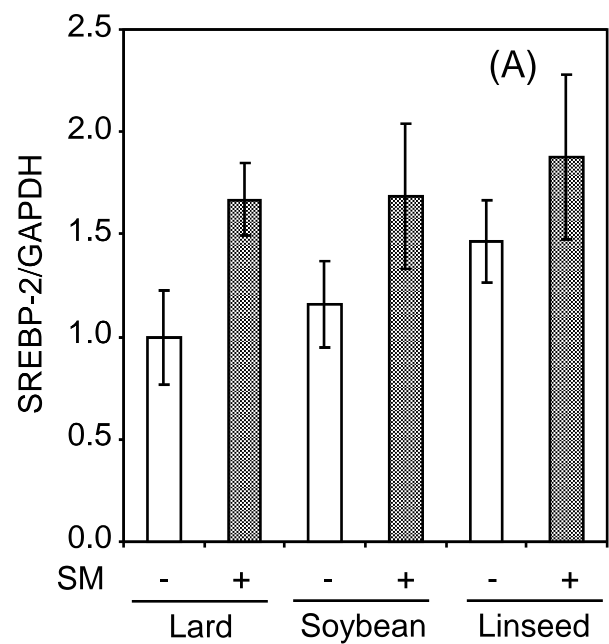
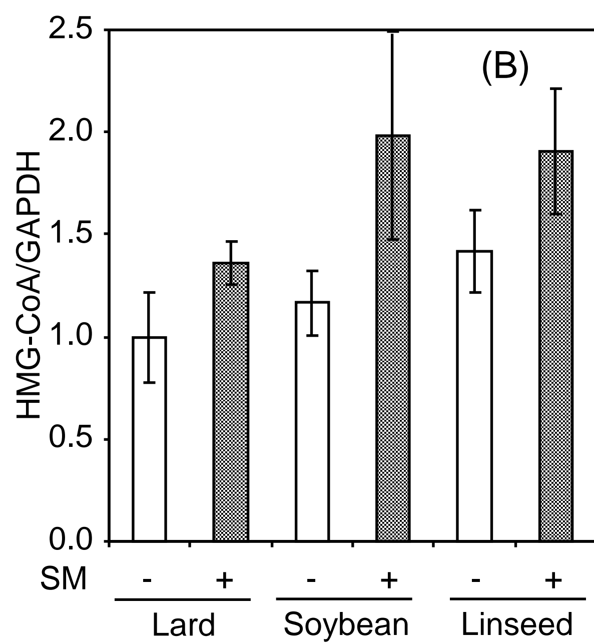


Fig. 7





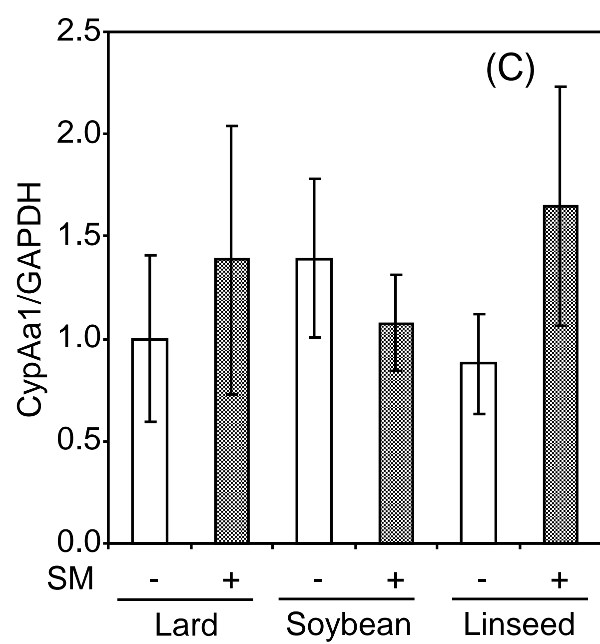
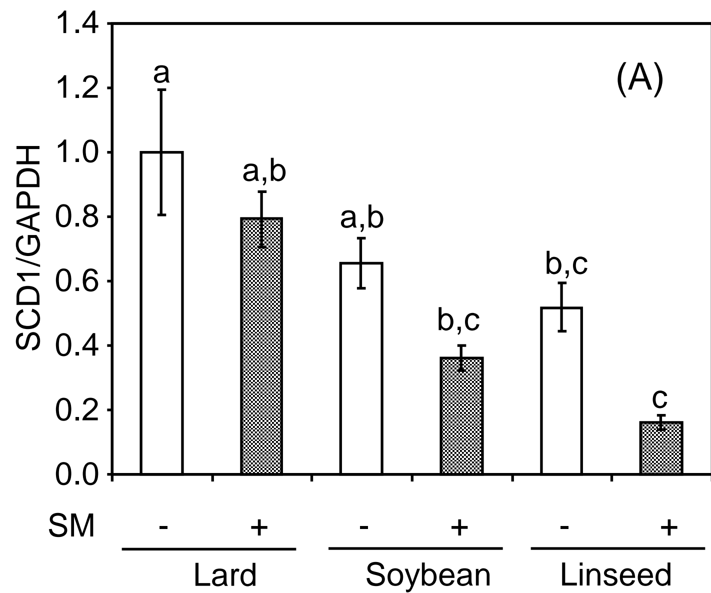
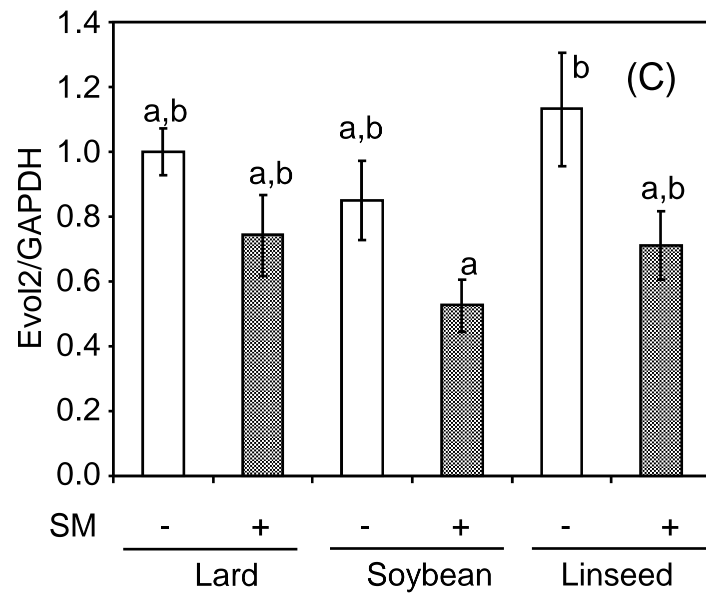
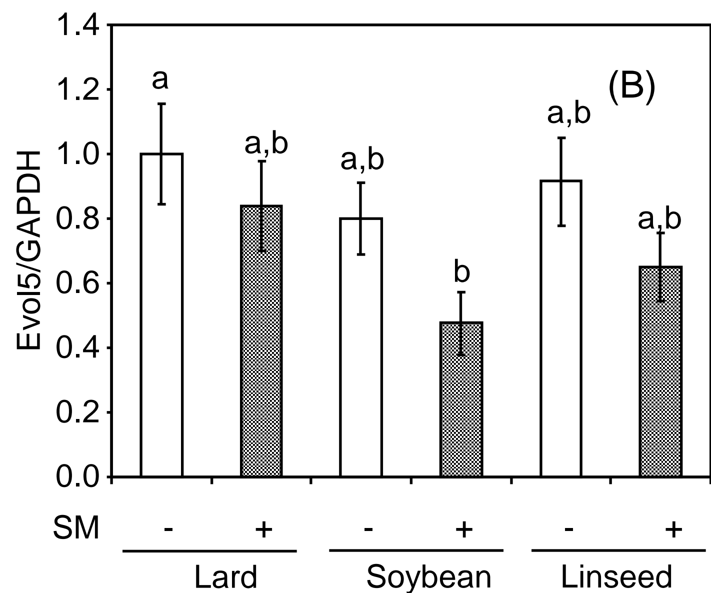


Fig. 8







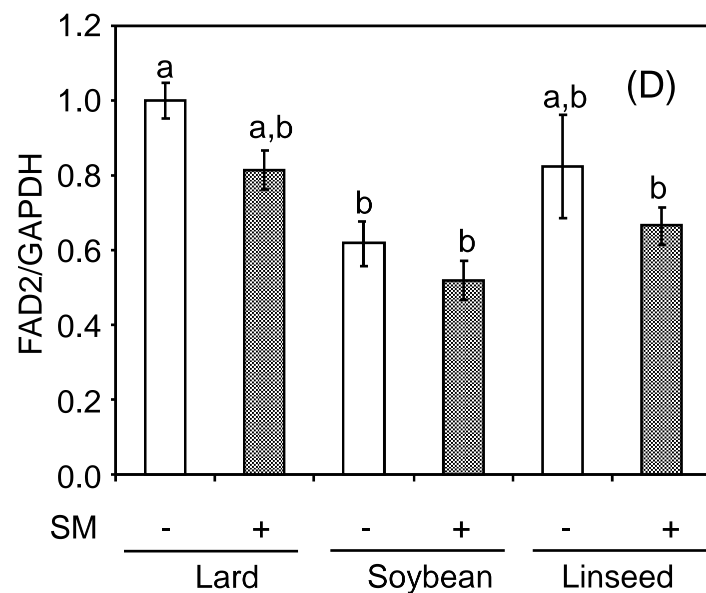


Fig. 9