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| Author(s)        | Kuroe, Miho; Kamogawa, Hiroyuki; Hosokawa, Masashi; Miyashita, Kazuo  |
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1 **Dietary Spinach ALA Enhances Liver n-3 Fatty Acid Content to Greater Extent**  
2 **Than Linseed Oil in Mice Fed Equivalent Amounts of ALA**

3  
4 Miho Kuroe, Hiroyuki Kamogawa, Masashi Hosokawa, and \*Kazuo Miyashita  
5 Laboratory of Bio-functional Material Chemistry, Division of Marine Bioscience,  
6 Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

7  
8 *Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido*  
9 *041-8611, Japan*

10  
11 \*Corresponding author: Tel.: +81 138 408804; fax: +81 138 408804  
12 E-mail address: kmiya@fish.hokudai.ac.jp

13  
14 **Abstract** Although several works have reported absorption rate differences of n-3  
15 polyunsaturated fatty acids (PUFA) bound to different lipid forms, such as ethyl ester,  
16 triacylglycerol (TAG), and phospholipids, no studies have investigated the effect of n-3  
17 PUFA from glycolipids (GL). The present study compared the fatty acid contents of  
18 tissue and serum lipids from normal C57BL/6J mice fed two types of  $\alpha$ -linolenic acid  
19 (ALA)-rich lipids, spinach lipid (SPL) and linseed oil (LO). ALA was primarily present  
20 as the GL form in SPL, while it existed as TAG in LO. Supplementations of both lipids  
21 increased ALA and its n-3 metabolites, eicosapentaenoic acid (EPA), docosapentaenoic  
22 acid (DPA), and docosahexaenoic acid, and decreased n-6 PUFA, linoleic acid and  
23 arachidonic acid, in the livers, small intestines, and sera of the treated mice compared  
24 with those of the control group. When the comparison between the SPL and LO diets  
25 containing the same amount of ALA was conducted, the EPA and DPA levels in the  
26 liver lipids from mice fed the SPL diet were significantly higher than those fed the LO  
27 diet. Additionally, the total contents of n-3 PUFA of lipids from the livers, small  
28 intestines, and sera of the SPL group were higher than those of the LO group.

29  
30 **Keywords** glycolipids,  $\alpha$ -linolenic acid absorption, eicosapentaenoic acid,  
31 docosapentaenoic acid, arachidonic acid

32  
33 **Abbreviations**

34 ALA  $\alpha$ -Linolenic acid  
35 ARA Arachidonic acid  
36 DGDG Digalactosyl diacylglycerol

|    |      |                                |
|----|------|--------------------------------|
| 37 | DHA  | Docosahexaenoic acid           |
| 38 | DPA  | Docosapentaenoic acid          |
| 39 | EE   | Ethyl esters                   |
| 40 | EPA  | Eicosapentaenoic acid          |
| 41 | FFA  | Free fatty acid                |
| 42 | GC   | Gas chromatography             |
| 43 | GL   | Galactolipids                  |
| 44 | LA   | Linoleic acid                  |
| 45 | LO   | Linseed oil                    |
| 46 | MGDG | Monogalactosyl diacylglycerol  |
| 47 | PC   | Phosphatidyl choline           |
| 48 | PL   | Phospholipids                  |
| 49 | SPL  | Spinach lipids                 |
| 50 | SQDG | Sulfoquinovosyl diacylglycerol |
| 51 | TAG  | Triacylglycerol                |
| 52 | TL   | Total lipids                   |
| 53 | TLC  | Thin layer chromatography      |

54  
55

## 56 **Introduction**

57

58 Plant leaves generally contain up to 7 weight % total lipids (TL) per dry weight [1]. We  
59 have analyzed TL levels of 13 types of commercial green leafy vegetables harvested in  
60 Hokkaido, Japan and found that the variation in the TL ranged from 6.1 - 13.0 weight %  
61 per dry weight [2]. This TL level was relatively higher than those of fruits, flowers, and  
62 stem vegetables (2 - 4 weight % per dry weight) (unpublished data). The higher TL level  
63 of green leafy vegetables is due to the large amounts of thylakoid membrane lipids in  
64 the tissues. The major constituents of leaf lipids are monogalactosyl diacylglycerol  
65 (MGDG) and digalactosyl diacylglycerol (DGDG) with moderate amounts of  
66 sulfoquinovosyl diacylglycerol (SQDG) and phospholipids (PL), while only small  
67 amounts of neutral lipids are present [3]. The high levels of MGDG and DGDG in the  
68 leaf lipids originate from the unusually high composition of both galactolipids (GL) in  
69 the thylakoid membranes of chloroplasts [4]. Usually, MGDG and DGDG from plant  
70 leaf lipids have very high amounts of  $\alpha$ -linolenic acid (18:3n-3, ALA) [1]. The ALA  
71 content of acylated fatty acids has been reported to be approximately 95% in MGDG  
72 from leaf chloroplasts of cucurbits [5], alfalfa [6], wheat [7], and green holly [8], while

73 that in DGDG has been reported in the range from 79-88%. Thus, green leafy vegetable  
74 lipids are potential dietary sources of ALA.

75 ALA is an essential fatty acid that must be consumed through the diet. There have  
76 been many epidemiological and clinical studies on the cardiovascular-protective effects  
77 of ALA [9]. ALA is a precursor of eicosapentaenoic (EPA; 20:5n-3) and  
78 docosahexaenoic (DHA; 22:6n-3) acids. Both n-3 EPA and DHA have sometimes been  
79 regarded as active forms of ALA in biological systems. EPA and DHA have been shown  
80 to cause significant biochemical and physiological changes in the body that often result  
81 in a positive influence on human nutrition and health. EPA and DHA consumptions  
82 have benefits of reducing the risk of cardiovascular disease, probably due to regulation  
83 of membrane structure, lipid metabolism, blood clotting, blood pressure, and  
84 inflammation [10-14]. Thus, the bioconversion of ALA to EPA and DHA is important  
85 for understanding the biological importance of ALA.

86 Humans have been generally considered to have a poor ability to form DHA from  
87 ALA. Tracer studies have shown that the proportion of ALA conversion to DHA in  
88 infants is very low, less than 1% [15]. Another study has demonstrated that in adult men,  
89 the conversion of ALA to EPA is limited (approximately 8%) and conversion to DHA is  
90 extremely low (<0.1%) [16]. However, studies in normal healthy adults consuming  
91 western diets showed that supplemental ALA raised EPA and DPA statuses in the blood  
92 and breast milk. Addition of ALA to the diets of formula-fed infants has been shown to  
93 raise DHA levels [17]. Another study showed that there was no difference in brain DHA  
94 accretion between rats fed ALA and DHA [18]. This was due to decreased DHA  
95 metabolism and an increased rate of DHA synthesis in rats fed ALA. The conversion of  
96 ALA to DHA by the liver and other specific DHA-requiring tissues, such as the brain,  
97 provides ample DHA when sufficient ALA has been consumed [19]. Thus, the need for  
98 ALA is extremely apparent because ALA is by far the predominant form of n-3 PUFA  
99 consumed in the typical Western and vegetarian diets [20].

100 ALA is present in notable amounts in plant sources, including green leafy  
101 vegetables and commonly-consumed oils, such as rapeseed and soybean oils.  
102 Additionally, ALA-rich oils, such as flaxseed oil, are commercially available. These  
103 ALA-containing products are a major source of n-3 PUFAs, especially in Western and  
104 vegetarian diets. ALA in seed oils exists as triacylglycerol (TAG), while in green leafy  
105 vegetables most ALA is bound to GLs, such as MGDG and DGDG. MGDG and DGDG  
106 digestions are known to be based on lipase hydrolysis of pancreatic juice, similarly as  
107 TAG digestion [21,22]; however, the ALA absorption as these GL may be different  
108 from that of TAG because several studies have reported different absorption rate of

109 EPA and DHA in different ester forms, such as TAG, PL, and ethyl esters (EE) [23-28].  
110 Among these, EPA and DHA of PL have been reported to show the highest  
111 bioavailabilities, followed by those of TAG and EE [25-27]. Higher absorption rate of  
112 EPA and DHA derived from PL have been reported in a human study using krill oil as a  
113 dietary lipid [24,28].

114 Although the intake of each PUFA from the different dietary form such as EE, TAG,  
115 and PL have been investigated, no study has been performed on those lipids bound to  
116 GL. Thus, in the present study, the intake of ALA from GL was compared with that  
117 from TAG using spinach leaf as a source of ALA-rich GL.

118

119

## 120 **Materials and Methods**

121

### 122 **Standards and chemicals**

123

124 Standard MGDG, DGDG, and SQDG were purchased from Lipid Products (Redhill,  
125 UK), while standard phosphatidyl choline (PC) was from Avanti Polar Lipids Inc.  
126 (Alabaster, AL, USA). All of the other chemicals and solvents used in this study were of  
127 analytical grade.

128

### 129 **Separation and analysis of spinach leaf lipids**

130

131 Dried spinach leaf powder (GABAN Co. Ltd., Tokyo, Japan) was obtained from a local  
132 food market. The spinach powder (2 kg) was extracted with 6 volumes (v/w) of  
133 methanol. The methanol extracts were dissolved into a separatory funnel using a  
134 chloroform/methanol/water (10:5:3, v/v/v) solution. After being shaken, the funnel was  
135 allowed to stand overnight, and the lower layer was concentrated under vacuum using a  
136 rotary evaporator. The last traces of organic solvent and water were removed in a  
137 desiccator under high vacuum to obtain spinach lipids (SPL). The lipid class profile of  
138 SPL was analyzed by thin layer chromatography (TLC). The lipid fraction was  
139 dissolved in a chloroform/methanol/water (65:25:4, v/v/v) solution and spotted onto  
140 0.25 mm silica gel plates (Merck, Darmstadt, Germany). The plates were developed  
141 with a chloroform-methanol-water (65:25:4, v/v/v) solution and the spots were  
142 visualized by spraying the plates with orcinol sulfuric acid or dittmer reagent, followed  
143 by charring. The lipid samples were also analyzed by silica gel TLC using  
144 *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as the developing solvent. The spot

145 was detected using 50% aqueous sulfuric acid charring. The chromatogram was taken  
146 with a charge coupled device camera, and then the digital image of the plate was  
147 acquired and transferred to the computer. The image was properly cropped and saved in  
148 bitmap format using Vistascan software on a Windows-controlled system  
149 (Hewlett–Packard, Tokyo, Japan). The ratio of each lipid fraction in the sample was  
150 expressed as the bitmap percentage of the total bitmap intensities.

151 The fatty acid compositions of SPL and linseed oil (LO) used as dietary lipids were  
152 determined by gas chromatography (GC) after converting fatty acyl groups in the lipids  
153 to their corresponding methyl esters by transesterification using sodium methoxide  
154 ( $\text{CH}_3\text{ONa}$ ) as the catalyst [29]. Briefly, 1 mL of *n*-hexane and 0.2 mL of 2N NaOH in  
155 methanol were added to an aliquot of sample lipids, vortexed and incubated at 50°C for  
156 30 seconds. Next, 0.2 mL of 2N HCl in methanol solution was added to the solution and  
157 mixed. The upper hexane layer was recovered and subjected to GC analysis. GC  
158 analysis was performed on a Shimadzu GC-14B instrument (Shimadzu Seisakusho,  
159 Kyoto, Japan) equipped with a flame-ionization detector and a capillary column  
160 [Omegawax 320 (30 m × 0.32 mm i.d.); Supelco, Bellefonte, PA]. The injection port  
161 and flame ionization detector were set at 250 and 260°C, respectively; the column  
162 temperature was maintained at 200°C. The carrier gas was helium at a flow rate of 50  
163 kPa. The fatty acid contents in lipid samples were expressed as the weight percentages  
164 of total fatty acids. For the reference, fresh leafy vegetables obtained in the local market  
165 in Hakodate, Japan, were extracted with chloroform/methanol/water (10:5:3, v/v/v) after  
166 freeze-dried treatment and the fatty acid composition of the lipids was analyzed as  
167 described above.

168

## 169 **Animals and diets**

170

171 The aim of this study was to compare the SPL and LO as dietary ALA source under  
172 normal conditions. Thus, normal and healthy C57BL/6J mice were used the rodent  
173 model. A total of 27 normal and healthy C57BL/6J mice (4-week old, male) were  
174 purchased from Charles River Laboratories (Japan, Inc., Yokohama, Kanagawa, Japan).  
175 All mice were housed in stainless cages (7 mice per a cage, 4 cages in total) and  
176 acclimatized for 2 weeks on a normal rodent diet MF (Oriental Yeast Co., Ltd., Tokyo,  
177 Japan). Mice had free access to food and tap water. Room temperature and humidity  
178 were controlled at  $23 \pm 1^\circ\text{C}$  and  $50 \pm 10\%$  with a 12 h light/12 h dark cycle. In most  
179 animal experiments using C57BL/6J mice, more than 6 mice were used. The mice were,  
180 therefore, randomly divided into 4 groups of 7 mice in one cage and then fed

181 experimental diets for 4 weeks. The body weight, diet and water intake of each mouse  
182 was recorded every day. The compositions of the diets are shown in Table 1. All dietary  
183 components except for lipids were obtained from CLEA (Japan, Inc. Tokyo, Japan).  
184 Lard was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Corn oil and LO  
185 were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). For the preparation  
186 of SPL containing diet, SPL and lard were dissolved in chloroform. After mixed well,  
187 the solvent was completely removed. The mix lipid was heated at 50°C, and then, the  
188 liquid was mixing with other dietary components. The two experimental diets, SPL  
189 (3%) and LO were designed to contain equal amounts of ALA, equating to  
190 approximately 0.7g/g of the diet (Table 2). However, the resulting manufactured diets  
191 had slightly different of total fatty acid intake because of the difference in the non-acyl  
192 component levels of SPL and LO. By the GC analysis using 17:0 as an internal standard,  
193 fatty acid content of the SPL (3%) diet was 5.2 g/7g lipids , while that of the LO diet  
194 was 6.8g/7g lipids. To All procedures for the use and care of animals for this research  
195 were approved by the Ethical Committee of Experimental Animal Care at Hokkaido  
196 University.

197

#### 198 **Fatty acid compositions of dietary lipids and ALA levels of each diet**

199

200 Dietary lipids were extracted from each diet with a chloroform/methanol (2:1, v/v)  
201 solution after being prepared, as described previously by Folch *et al* [30]. The  
202 chloroform/methanol solution contained a known amount of 17:0 as an internal standard.  
203 Fatty acid compositions of the lipids were analyzed by GC after converting fatty acyl  
204 groups in the lipids to their methyl esters, as described above. The ALA levels of the  
205 diets were calculated by comparing the peak ratios of ALA to that of the internal  
206 standard (17:0) and the lipid content.

207

#### 208 **Sample collections**

209

210 Blood samples were taken from the caudal vena cava of the mice. Mice were euthanized,  
211 and each tissue was immediately excised and weighed. The livers were immediately  
212 stored in RNA later™ (Sigma Chemical Co., St. Louis, MO) for quantitative real-time  
213 PCR analysis. Blood serum analyses were conducted by the Analytical Center of  
214 Hakodate Medical Association (Hakodate, Japan). The analyses included measuring the  
215 following parameters: neutral lipids, free fatty acids, phospholipids, total cholesterol,  
216 HDL cholesterol, LDL cholesterol, and free fatty acids.

217

## 218 **Tissue and blood lipid analysis**

219

220 Livers, small intestines, and brains were extracted with a chloroform/methanol (2:1,  
221 v/v) solution containing a known amount of internal standard (17:0), as described  
222 previously by Folch *et al* [30]. The tissue samples from each mouse were analyzed  
223 separately. The major fatty acid contents of each tissue were analyzed by GC after  
224 converting fatty acyl groups in the lipid to their methyl esters, as described above. The  
225 contents were reported as mg per gram tissue. However, only small amounts of serum  
226 samples remained after the lipid parameter analyses. Therefore, all serum samples were  
227 combined in each group, and then the lipids were extracted with a  
228 chloroform/methanol/water (1:2:0.8, v/v/v) solution, as described previously by Bligh  
229 and Dyer [31]. Fatty acid compositions (weight % of total fatty acids) were analyzed by  
230 GC after converting fatty acyl groups in the lipid to their methyl esters, as described  
231 above.

232

## 233 **Quantitative Real-Time PCR**

234

235 Total RNA was extracted from the livers of mice using RNeasy Lipid Tissue Mini Kits  
236 (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then  
237 synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kits  
238 (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real-time PCR analyses of  
239 individual cDNA were performed with ABI Prism 7500 (Applied Biosystems Japan Ltd.,  
240 Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.,  
241 Tokyo, Japan). The mRNA analyses were performed on genes associated with the  
242 bioconversion of ALA to DHA, which included  $\Delta^6$ -desaturase (Fads2) (from ALA to  
243 18:4n-3 and 24:5n-3 to 24:6n-3), elongase-5 (Elov5) (from 18:4n-3 to 20:4n-3),  
244  $\Delta^5$ -desaturase (Fads1) (from 20:4n-3 to EPA), elongase-2 (Elov2) (from EPA to DPA  
245 and DPA to 24:5n-3), and peroxisomal acyl CoA oxidase (Acox1) (from 24:6n-3 to  
246 DHA). The gene-specific primers Mm00507605\_m1 (Fads1 mRNA),  
247 Mm00517221\_m1 (Fads2 mRNA), Mm00517086\_m1 (Elov2 mRNA),  
248 Mm00506717\_m1 (Elov5 mRNA), Mm01246834\_m1 (Acox1 mRNA), and  
249 Mm99999915\_g1 (GAPDH mRNA; internal control), respectively.

250

## 251 **Statistical analysis**

252



253 Data are presented as the means  $\pm$  SEM (n=6 or 7). Analysis of variance (ANOVA) was  
254 used to test for significant differences between different groups. Statistical comparisons  
255 were performed using Scheffe's *F*-test. Differences with  $P < 0.05$  were considered  
256 significant.

257

258

## 259 **Results**

260

### 261 **Fatty acid contents of dietary lipids**

262

263 The major fatty acids of SPL were ALA (53.2%), 16:0 (13.9%), and linoleic acid  
264 (18:2n-6, LA) (12.3%). Analysis of other leafy vegetable lipids showed the highest  
265 content of ALA in the fatty acids: Komatsuna (*Brassica rapa* var. *perviridis*), 52.5%;  
266 Mizuna (*Brassica rapa* var. *laciniifolia*), 50.8%, Perilla (*Perilla frutescens* var. *acuta*)  
267 52.3%; Sweet basil (*Ocimum basilicum*), 56.8%; Mistuba (*Cryptotaenia japonica*),  
268 35.0; Parsley (*Petroselinum crispum*), 30.5%; Garland chrysanthemum  
269 (*Chrysanthemum coronarium*), 68.3%; Garlic chives (*Allium tuberosum*) 48.6%; Welsh  
270 onion (*Allium fistulosum*) 47.2%. These leafy vegetables contained 6.1-13.0 weight %  
271 lipids per dry weight and most of lipids composed of GL rich in ALA.

272 LO also contained a high level of ALA (45.3%), followed by 18:1n-9 (25.3%), LA  
273 (16.3%), and 16:0 (5.9%). However, the lipid class compositions of LO and SPL were  
274 different. TLC analysis showed that LO was mainly composed of TAG; however, only a  
275 little amount of TAG was detected in SPL. When each lipid composition of SPL was  
276 roughly analyzed based on the spot intensities of TLC, the main lipid class of SPL was  
277 found to be DGDG (22.0%), followed by MGDG (17.3%), SQDG (13.5%),  
278 chlorophylls (11.6%), PC (3.5%), and lutein (1.3%). Chlorophylls and lutein are  
279 non-acyl lipids. MGDG, DGDG, SQDG, and PC are diacyl glycerols with non-acyl  
280 components bound to the remaining position of glycerol, resulting in the relatively  
281 lower percentage of fatty acids in SPL than in LO. Therefore, for the comparison of the  
282 dietary SPL with that from LO, LO (15 g/1 kg diet) was added to the diet so that the  
283 ALA level of the diet was almost the same as that in the diet containing SPL (3%)  
284 (Table 2).

285

286 **Fatty acid levels of livers (Table 3), small intestines (Table 4), brains (Table 5), and**  
287 **sera (Table 6)**

288

289 All animals remained healthy throughout the experimental period. There were no  
290 significant differences in the body weights, food and water intakes, liver, small intestine,  
291 brain, muscle, heart, or kidney weights of mice fed four types of diets. Food intake (g)  
292 per day of each group was  $24.16 \pm 2.14$ ,  $24.13 \pm 2.63$ ,  $23.45 \pm 2.53$ ,  $23.80 \pm 3.99$  for control,  
293 LO, SPL (1%) and SPL (2%) group, respectively. There was also no significant  
294 difference in dairy food intake among different four groups. Supplementation of  
295 ALA-rich diets, including LO, SPL (1%) and SPL (3%) significantly increased hepatic  
296 n-3 PUFA levels, including ALA, EPA, and docosapentaenoic acid (DPA, 22:5n-3),  
297 compared with the control, while a significant decrease in n-6 arachidonic acid (ARA,  
298 20:4n-6) was found in the ALA-rich diet feedings (Table 3). The LA level of mice fed  
299 ALA-rich diets also decreased, although the LA content in the ALA diets was higher  
300 than that in the control (Table 2). Mice fed LO and SPL (3%) showed significantly  
301 higher DHA levels relative to the control. The DHA level in mice fed SPL (1%) also  
302 increased, but the difference compared with the control was not significant.

303 Although the ALA content of the diet was the same for LO and SPL (3%) (Table 2),  
304 significantly higher contents of hepatic EPA and DPA were found in mice fed SPL (3%)  
305 than in the LO group (Table 3). Additionally, the ALA level of the mice fed SPL (3%)  
306 was higher than those fed LO, but the difference was not significant. The higher ALA  
307 level in the mice fed SPL (3%) than those fed LO was also found in the small intestinal  
308 lipids (Table 4) and in the serum lipids (Table 5). In serum lipids, EPA and DPA were  
309 also much higher in mice fed SPL (3%) than in the LO group. However, the difference  
310 in the fatty acid contents in brain lipids was small (Table 6).

311 Although there were no significant differences in serum total cholesterol (Fig. 1A),  
312 LDL cholesterol (Fig. 1C), neutral lipids (Fig. 1D), PL (Fig. 1E), and free fatty acid  
313 (FFA) (Fig. 1F) levels among the four dietary groups, HDL cholesterol (Fig. 1B)  
314 significantly increased in mice fed SPL (1%) and SPL (3%).

315

### 316 **Gene expression of elongase, desaturase and peroxisomal enzymes involved in ALA** 317 **bioconversion to DHA**

318

319 ALA is converted to DHA through a series of desaturation and chain elongation  
320 processes. Fig. 2 shows the effect of the experimental diets on the relative mRNA  
321 expression levels of these fatty acids desaturase, elongase, and acyl CoA oxidase in the  
322 liver. SPL (3%) supplementation significantly decreased *Fads1* and *Fads2*, while a  
323 significant increase in *Acox1* was found in the SPL (3%) group. However, LO had no  
324 significant effect on these gene expressions compared with the control group.

325

## 326 **Discussion**

327

328 Supplementations of ALA-rich lipids, LO and SPL resulted in increased ALA and its  
329 n-3 metabolites (EPA, DPA, and DHA) and decreased n-6 PUFA (LA and ARA) in  
330 mice livers, small intestines, and sera (Tables 3, 4, and 5). Increased n-3 PUFA and  
331 decreased n-6 PUFA in rat organs, including the brain, as a result of ALA feeding have  
332 been reported in other studies [32-34]; however, in the present study, little effect on the  
333 fatty acid composition of brain lipids was observed (Table 6). Lipid and fatty acid  
334 compositions of the brain are usually less affected by dietary lipids in normal conditions  
335 and strictly regulated through DHA uptake from the plasma and brain DHA metabolism  
336 [18]. Therefore, the result in Table 6 may be due to homeostasis found in normal  
337 C57BL/6J mice administered the experimental diet for only 4 weeks in the present study.  
338 The same result has also been obtained in female ddy mice fed DHA-rich lipids [26].  
339 They reported that the DHA levels of the serum and the liver lipids were significantly  
340 increased by feeding DHA lipids, but the fatty acid composition of the brain did not  
341 change drastically.

342 Although the ALA contents in the LO and SPL (3%) diets were the same (Table 2),  
343 the EPA and DPA levels in the liver lipids from the mice fed the SPL (3%) diet were  
344 significantly higher than those fed the LO diet (Tables 3 and 5). Additionally, the ALA  
345 level of lipids from the livers, small intestines, brains, and plasmas of mice fed the SPL  
346 (3%) diet were also higher than those fed the LO diet (Tables 3, 4 and 5). These results  
347 suggest that ALA originated from SPL may be absorbed more efficiently than that from  
348 LO. However, a significant increase in EPA and DPA levels of hepatic lipids from mice  
349 fed SPL (3%), shown in Table 2, may be derived from up-regulation of ALA  
350 bioconversion to EPA and DPA. This reaction is regulated by the activities of different  
351 enzymes, including  $\Delta^6$ -desaturase (Fads2), elongase-5 (Elov5),  $\Delta^5$ -desaturase (Fads1),  
352 and elongase-2 (Elov2). However, the gene expressions of Fads2 and Fads1  
353 significantly decreased in the SPL fed group (Fig. 2). Additionally, SPL feeding had no  
354 significant effect on both elongases, showing little effect of SPL feeding on  
355 up-regulating bioconversion of ALA to EPA and DPA.

356 In LO, greater than 99% of ALA was present as TAG, whereas most of ALA in SPL  
357 was incorporated into GLs, such as MGDG or DGDG [3,5-8]. We have reported 75.2  
358 and 77.2% ALA in MGDG and DGDG from spinach powder lipids, respectively [35].  
359 Both GL were the major lipid components of SPL, and a high ALA level (53.2%) was  
360 found in the SPL used in the present study. The higher levels of ALA and of its

361 metabolites, EPA and DPA, in the mice fed the SPL (3%) diet was probably due to the  
362 higher absorption rate of ALA in the MGDG and/or DGDG forms from an intestinal  
363 part.

364 Several studies have demonstrated the absorption efficacies of n-3 PUFA from  
365 different chemical forms [25,36]. Generally, PL has been considered to be better  
366 absorbed than the TAG form, especially in infants [25]. A comparative study using free  
367 fatty acids (FFA), EE, TAG, and PL forms of DHA showed that DHA-PL was more  
368 effective at increasing DHA in the liver and the brain of male Balb/c mice than other  
369 DHA forms [27]. A human study on the uptake of n-3 PUFA as PL form has been  
370 reported using krill oil containing 30 - 65% of the fatty acids as the PL form. In a  
371 double-blinded crossover trial, Schuchardt *et al* [24] compared the uptake of  
372 EPA+DHA from three different oral administrations of fish oil TAG, EE, and krill oil  
373 (mainly PL). Although the intake levels of EPA and DHA were the same among the  
374 three groups, the krill oil group showed the highest incorporation of EPA+DHA into  
375 plasma, followed by TAG and EE.

376 The amphiphilic character of PL has been proposed as the most likely reason for the  
377 higher intake of EPA+DHA from PL compared with that from TAG [24]. ALA bound  
378 to GL is also due to the amphiphilic character of this lipid, similarly to PL. GL  
379 possesses emulsification properties due to the presence of a galactosyl group. As a  
380 result, GL has been shown to influence the surface composition of fat droplets and  
381 increase the binding rate of hydrolyzing enzymes [37]. This surfactant ability of GL has  
382 also been shown to promote the formation of mixed micelles and therefore the digestion.  
383 The first step in GL digestion is hydrolysis of the *sn*-1 position on the glycerol backbone  
384 by pancreatic lipase, yielding monoacylgalactolipids [22]. This lyso compound shows  
385 higher emulsification properties than the corresponding diacylglycerols from TAG. Thus,  
386 it may be possible that the high GL level in SPL leads to an enhanced absorption of  
387 lipids rich in ALA. However, the present study only analyzed fatty acid distribution of  
388 several tissues and sera. This study has a small sample size and was short in duration  
389 (only 4 weeks). In addition there were no measurements of n-3 PUFA excretion and no  
390 direct measurement of n-3 PUFA intake. While, we did measure fatty acids in serum,  
391 liver, brain and intestine, several tissues were not examined including the heart and  
392 skeletal muscle that can accumulate and serve as deposits of n-3 PUFA [38]. Longer  
393 experiments in different types of animals to ensure fatty acid levels reach equilibrium  
394 and a comprehensive fatty acid analysis of blood lipids (plasma, erythrocytes, or  
395 leukocytes) and other tissues are needed. For further insight on absorption and  
396 metabolism of n-3 PUFA from glycolipids, intake and excretion measurements as well

397 as tracer studies using isotope-labeled ALA are needed [39-41].

398 Numerous epidemiological studies, clinical trials, genetic and nutrigenetic  
399 approaches have demonstrated the health benefits of n-3 PUFA, such as ALA, EPA, and  
400 DHA. The most apparent benefit of these n-3 PUFA is reduction of cardiovascular risk,  
401 probably due to regulation of membrane structure, lipid metabolism, blood clotting,  
402 blood pressure, and inflammation [42]. Dietary ALA has been known to improve blood  
403 lipid levels [9,19]. However, there was no significant effect of dietary ALA on plasma  
404 total cholesterol, LDL cholesterol, neutral lipids, PL, and FFA levels found in the  
405 present study using normal mice fed normal diets, although a decreasing trend in neutral  
406 lipids, FFA, and LDL cholesterol was observed in ALA-containing diets (Figure 1).  
407 However, significant increases in HDL cholesterol were found in the mice fed SPL  
408 (1%) and (3%) diets (Figure 1). Although the reason for the higher HDL levels is  
409 unknown, the effect of SPL should be attributed to the biological activities of the  
410 characteristic lipid constituents of SPL containing MGDG, DGDG, and SQDG as major  
411 components. Studies on both synthetic and natural MGDG and DGDG have revealed  
412 their activities, including anti-tumor, anti-inflammatory, and cell cycle regulation  
413 [43-45]; however, no studies regarding the effect of GL on lipid metabolism have been  
414 conducted. SPL also contained lutein as a major carotenoid. Lutein has attracted great  
415 attention for preventing and reversing certain serious eye diseases [46,47]; however,  
416 little is known about the effect of lutein on lipid metabolism. More studies may need to  
417 be conducted on the biological effects of these leafy lipid components.

418 In conclusion, the present study demonstrated the important role of green leafy  
419 vegetables as n-3 sources. Leafy vegetable lipids, such as SPL, are mainly composed of  
420 DGDG and MGDG rich in ALA. A wide range of plant products contain ALA,  
421 including seeds, nuts, vegetables, legumes, grains, and fruits. Among them, several  
422 types of seed oils, such as LO, flaxseed oil and walnut oil, are known to be ALA  
423 sources. In addition to these seed oils, green leafy vegetable lipids should also be  
424 considered because they are rich in ALA as GL forms and in phytochemicals, such as  
425 carotenoids.

426

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430

431 **Conflict of interest** The authors declare that there are no conflicts of interest.

432

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565

566 Figure legends

567

568 **Figure 1.** Effects of dietary lipids on serum lipid parameters of C57BL/6J mice. (A),  
569 Total cholesterol; (B), HDL cholesterol; (C), LDL cholesterol; (D), neutral lipids; (E),  
570 phospholipids (PL); (F), free fatty acids (FFA). Values represent means  $\pm$  SE of seven  
571 mice per group. Different letters show significant differences at  $P < 0.05$ .

572

573 **Figure 2.** Expression of mRNA of genes associated with the bioconversion of  
574  $\alpha$ -linolenic acid (ALA) to docosahexaenoic acid (DHA) in C57BL/6J mice fed different  
575 dietary lipids. Values represent means  $\pm$  SE of seven mice per group. Different letters  
576 show significant differences at  $P < 0.05$ .

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

| Diet ingredient             | Group   |         |          |          |
|-----------------------------|---------|---------|----------|----------|
|                             | Control | LO      | SPL (1%) | SPL (3%) |
| Corn starch                 | 397.486 | 397.486 | 397.486  | 397.486  |
| Dextrinized corn starch     | 132     | 132     | 132      | 132      |
| Casein                      | 200     | 200     | 200      | 200      |
| Sucrose                     | 100     | 100     | 100      | 100      |
| Cellulose (KC flock)        | 50      | 50      | 50       | 50       |
| AIN93G mineral mix          | 35      | 35      | 35       | 35       |
| AIN93G vitamin mix          | 10      | 10      | 10       | 10       |
| L-cystine                   | 3       | 3       | 3        | 3        |
| Choline bitartrate          | 2.5     | 2.5     | 2.5      | 2.5      |
| <i>t</i> -Butylhydroquinone | 0.014   | 0.014   | 0.014    | 0.014    |
| Lard                        | 50      | 55      | 60       | 40       |
| Corn oil                    | 20      | 0       | 0        | 0        |
| Linseed oil (LO)            | 0       | 15      | 0        | 0        |
| SPL                         | 0       | 0       | 10       | 30       |

Spinach leaf lipids (SPL) was obtained from dried spinach leaf powder by solvent extraction described in the Materials and Methods section. It mainly composed of galactolipids (GL).

**Table 2** Fatty acid content of total fatty acids and total diet

|                           | Group   |       |          |          |
|---------------------------|---------|-------|----------|----------|
|                           | Control | LO    | SPL (1%) | SPL (3%) |
| Wt% per total fatty acids |         |       |          |          |
| 14:0                      | 1.14    | 1.28  | 1.46     | 1.22     |
| 16:0                      | 21.44   | 21.57 | 24.67    | 23.10    |
| 18:0                      | 10.25   | 11.45 | 12.75    | 10.59    |
| 18:1n-9                   | 38.85   | 37.56 | 39.44    | 33.03    |
| 18:1n-7                   | 2.25    | 2.48  | 2.69     | 2.39     |
| 18:2n-6                   | 2.02    | 9.40  | 7.92     | 8.74     |
| 18:3n-3                   | 0.63    | 10.27 | 3.99     | 12.71    |
| Grams per 100g diet       |         |       |          |          |
| 14:0                      | 0.08    | 0.09  | 0.09     | 0.06     |
| 16:0                      | 1.50    | 1.46  | 1.55     | 1.21     |
| 18:0                      | 0.72    | 0.78  | 0.80     | 0.09     |
| 18:1n-9                   | 2.72    | 2.55  | 2.48     | 0.56     |
| 18:1n-7                   | 0.16    | 0.17  | 0.17     | 1.73     |
| 18:2n-6                   | 1.41    | 0.64  | 0.50     | 0.13     |
| 18:3n-3                   | 0.04    | 0.70  | 0.25     | 0.67     |

**Table 3** TL level and fatty acid content of liver

|                             | Group                    |                          |                          |                         |
|-----------------------------|--------------------------|--------------------------|--------------------------|-------------------------|
|                             | Control                  | LO                       | SPL (1%)                 | SPL (3%)                |
| Liver weight<br>(g/100g BW) | 4.67±0.28                | 4.56±0.24                | 4.86±0.13                | 4.99±0.09               |
| TL (mg/g tissue)            | 56.98±6.42               | 55.72±4.38               | 58.21±3.86               | 57.08±4.98              |
| Fatty acid (µmol/g tissue)  |                          |                          |                          |                         |
| 16:0                        | 32.22±15.83              | 30.69±5.19               | 29.38±2.78               | 29.05±2.56              |
| 16:1                        | 4.74±2.73                | 5.71±1.23                | 5.83±1.13                | 5.93±0.79               |
| 18:0                        | 12.19±5.86               | 10.19±0.95               | 9.73±0.59                | 9.81±1.07               |
| 18:1n-9                     | 36.14±25.34              | 36.76±13.11              | 33.69±4.61               | 27.33±3.39              |
| 18:1n-7                     | 5.04±2.87                | 4.09±0.97                | 4.87±0.70                | 3.77±0.67               |
| 18:2n-6                     | 15.76±7.93 <sup>b</sup>  | 9.79±1.60 <sup>a,b</sup> | 9.07±0.70 <sup>a</sup>   | 8.39±1.00 <sup>a</sup>  |
| 18:3n-3                     | 0.11±0.06 <sup>a</sup>   | 1.66±0.38 <sup>c</sup>   | 0.64±0.09 <sup>b</sup>   | 1.79±0.27 <sup>c</sup>  |
| 20:3n-6                     | 2.08±1.04 <sup>b</sup>   | 1.30±0.32 <sup>a,b</sup> | 1.41±0.10 <sup>a,b</sup> | 1.13±0.12 <sup>a</sup>  |
| 20:4n-6                     | 14.16±7.08 <sup>b</sup>  | 6.51±0.57 <sup>a</sup>   | 7.80±0.73 <sup>a</sup>   | 5.68±0.70 <sup>a</sup>  |
| 20:5n-3                     | 0.07±0.07 <sup>a</sup>   | 2.30±0.45 <sup>c</sup>   | 1.19±0.21 <sup>b</sup>   | 3.28±0.32 <sup>d</sup>  |
| 22:5n-3                     | 0.09±0.10 <sup>a</sup>   | 0.98±0.27 <sup>c</sup>   | 0.58±0.12 <sup>b</sup>   | 1.26±0.15 <sup>d</sup>  |
| 22:6n-3                     | 4.29±2.04 <sup>a</sup>   | 8.62±2.52 <sup>b</sup>   | 7.02±0.85 <sup>a,b</sup> | 7.96±1.17 <sup>b</sup>  |
| Total saturated             | 44.31±21.81              | 41.83±6.18               | 39.96±3.16               | 39.70±3.65              |
| Total monounsaturated       | 47.04±31.66              | 47.47±15.24              | 45.26±6.35               | 37.66±4.69              |
| Total n-6                   | 32.81±16.29 <sup>b</sup> | 18.02±2.53 <sup>a</sup>  | 18.67±1.49 <sup>a</sup>  | 15.50±1.83 <sup>a</sup> |
| Total n-3                   | 4.57±2.08 <sup>a</sup>   | 13.88±2.88 <sup>c</sup>  | 9.48±1.14 <sup>b</sup>   | 14.47±1.86 <sup>c</sup> |

Different letters show significantly different at  $P < 0.05$ .

**Table 4** TL level and fatty acid content of small intestine

|                                       | Group                  |                          |                          |                          |
|---------------------------------------|------------------------|--------------------------|--------------------------|--------------------------|
|                                       | Control                | LO                       | SPL (1%)                 | SPL (3%)                 |
| Small intestine weight<br>(g/100g BW) | 3.32±0.28              | 3.46±0.16                | 3.00±0.07                | 3.43±0.16                |
| TL (mg/g tissue)                      | 23.97±5.49             | 17.07±2.53               | 23.87±4.55               | 23.18±5.59               |
| Fatty acid (µmol/g tissue)            |                        |                          |                          |                          |
| 16:0                                  | 9.61±5.32              | 8.06±1.74                | 10.24±3.31               | 9.53±5.17                |
| 18:0                                  | 3.02±1.06              | 2.71±0.58                | 2.62±0.81                | 2.06±0.95                |
| 18:1n-9+18:1n-7                       | 15.30±9.43             | 12.73±2.60               | 18.29±6.61               | 14.83±8.35               |
| 18:2n-6                               | 4.68±2.21 <sup>b</sup> | 2.72±0.36 <sup>a,b</sup> | 2.79±0.88 <sup>a,b</sup> | 2.36±1.23 <sup>a</sup>   |
| 18:3n-3                               | 0.09±0.05 <sup>a</sup> | 0.77±0.16 <sup>b,c</sup> | 0.43±0.13 <sup>a,b</sup> | 1.04±0.57 <sup>c</sup>   |
| 20:4n-6                               | 0.91±0.27 <sup>b</sup> | 0.58±0.14 <sup>a,b</sup> | 0.56±0.26 <sup>a</sup>   | 0.37±0.19 <sup>a</sup>   |
| 20:5n-3                               | 0.00±0.00 <sup>a</sup> | 0.17±0.05 <sup>c</sup>   | 0.08±0.04 <sup>a,b</sup> | 0.15±0.08 <sup>b,c</sup> |
| 22:5n-3                               | 0.02±0.01 <sup>a</sup> | 0.14±0.03 <sup>b</sup>   | 0.08±0.04 <sup>a,b</sup> | 0.16±0.08 <sup>b</sup>   |
| 22:6n-3                               | 0.28±0.11 <sup>a</sup> | 0.51±0.10 <sup>b</sup>   | 0.39±0.16 <sup>a,b</sup> | 0.35±0.18 <sup>a,b</sup> |
| Saturated                             | 13.21±6.54             | 11.25±2.19               | 13.49±4.18               | 12.18±6.37               |
| Monounsaturated                       | 18.31±11.36            | 15.46±3.27               | 22.26±8.08               | 18.64±10.64              |
| Total n-6                             | 5.80±2.28 <sup>b</sup> | 3.48±0.46 <sup>a,b</sup> | 3.55±1.17 <sup>a,b</sup> | 2.85±1.45 <sup>a</sup>   |
| Total n-3                             | 0.38±0.14 <sup>a</sup> | 1.59±0.18 <sup>b</sup>   | 0.98±0.32 <sup>a,b</sup> | 1.70±0.87 <sup>b</sup>   |

Different letters show significantly different at  $P < 0.05$ .

**Table 5** Fatty acid composition of serum (wt % of total fatty acids)

|                 | Group   |       |          |          |
|-----------------|---------|-------|----------|----------|
|                 | Control | LO    | SPL (1%) | SPL (3%) |
| Fatty acid      |         |       |          |          |
| 16:0            | 24.82   | 25.24 | 24.36    | 25.60    |
| 16:1            | 3.11    | 2.95  | 3.42     | 3.70     |
| 18:0            | 10.68   | 12.19 | 11.48    | 10.95    |
| 18:1n-9         | 23.50   | 23.81 | 26.56    | 22.97    |
| 18:1n-7         | 3.42    | 2.77  | 3.58     | 2.93     |
| 18:2n-6         | 16.78   | 13.45 | 12.40    | 13.22    |
| 18:3n-6         | 0.16    | 0.08  | 0.09     | 0.09     |
| 18:3n-3         | 0.09    | 1.13  | 0.66     | 1.86     |
| 18:4n-3         | ND      | 0.04  | 0.02     | 0.04     |
| 20:3n-6         | 1.89    | 1.46  | 1.54     | 1.26     |
| 20:4n-6         | 7.41    | 3.60  | 4.14     | 3.31     |
| 20:5n-3         | 0.22    | 1.71  | 0.84     | 2.52     |
| 22:5n-3         | 0.09    | 0.68  | 0.42     | 1.01     |
| 22:6n-3         | 2.63    | 6.00  | 4.63     | 5.75     |
| Saturated       |         |       |          |          |
| Monounsaturated |         |       |          |          |
| Total n-6       | 26.24   | 18.59 | 18.17    | 17.88    |
| Total n-3       | 3.03    | 9.56  | 6.57     | 11.18    |

ND: Not detected.

**Table 6** TL level and fatty acid content of brain

|                            | Group                  |                          |                          |                          |
|----------------------------|------------------------|--------------------------|--------------------------|--------------------------|
|                            | Control                | LO                       | SPL (1%)                 | SPL (3%)                 |
| Brain weight (g/100g BW)   | 1.63±0.05              | 1.62±0.04                | 1.66±0.04                | 1.63±0.03                |
| TL (mg/g tissue)           | 69.01±2.40             | 67.81±3.34               | 64.79±2.49               | 71.66±1.41               |
| Fatty acid (µmol/g tissue) |                        |                          |                          |                          |
| 16:0                       | 21.78±1.97             | 21.06±3.19               | 21.43±2.40               | 23.39±1.29               |
| 18:0                       | 17.23±1.85             | 16.20±3.12               | 16.68±1.75               | 18.38±0.81               |
| 18:1n-9                    | 15.60±1.90             | 15.62±1.65               | 14.33±1.99               | 16.42±1.40               |
| 18:1n-7                    | 3.66±0.34              | 3.41±0.42                | 3.23±0.44                | 3.59±0.31                |
| 18:2n-6                    | 0.47±0.08 <sup>b</sup> | 0.08±0.05 <sup>b</sup>   | 0.32±0.05 <sup>a</sup>   | 0.38±0.01 <sup>a,b</sup> |
| 20:3n-6                    | 0.37±0.08              | 0.44±0.04                | 0.39±0.08                | 0.46±0.06                |
| 20:4n-6                    | 7.44±0.99              | 6.68±1.49                | 6.97±0.73                | 7.33±0.85                |
| 20:5n-3                    | ND                     | 0.02±0.04                | 0.02±0.05                | 0.04±0.06                |
| 22:5n-3                    | ND <sup>a</sup>        | 0.12±0.12 <sup>a,b</sup> | 0.05±0.10 <sup>a,b</sup> | 0.18±0.11 <sup>b</sup>   |
| 22:6n-3                    | 10.60±1.61             | 11.09±2.02               | 10.82±1.18               | 12.04±1.00               |
| Saturated                  | 39.49±3.78             | 37.70±6.06               | 38.52±4.06               | 42.21±1.96               |
| Monounsaturated            | 19.26±2.23             | 19.03±2.04               | 17.57±2.43               | 20.02±1.70               |
| Total n-6                  | 8.34±1.13              | 7.63±1.54                | 7.73±0.83                | 8.23±0.88                |
| Total n-3                  | 10.60±1.61             | 11.23±2.02               | 10.89±1.26               | 12.26±1.10               |

ND: not detected.

Different letters show significantly different at  $P < 0.05$ .

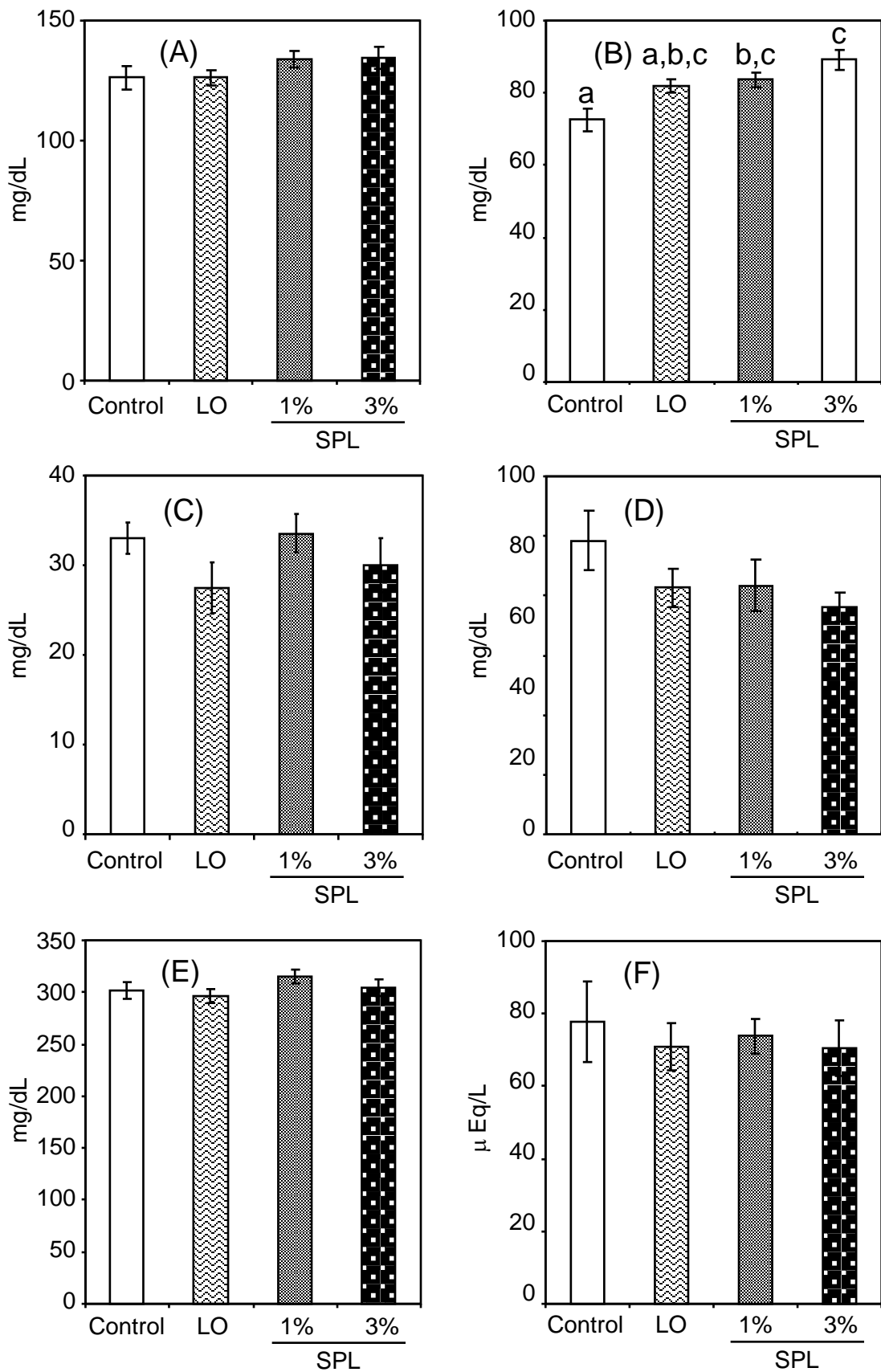


Fig. 1



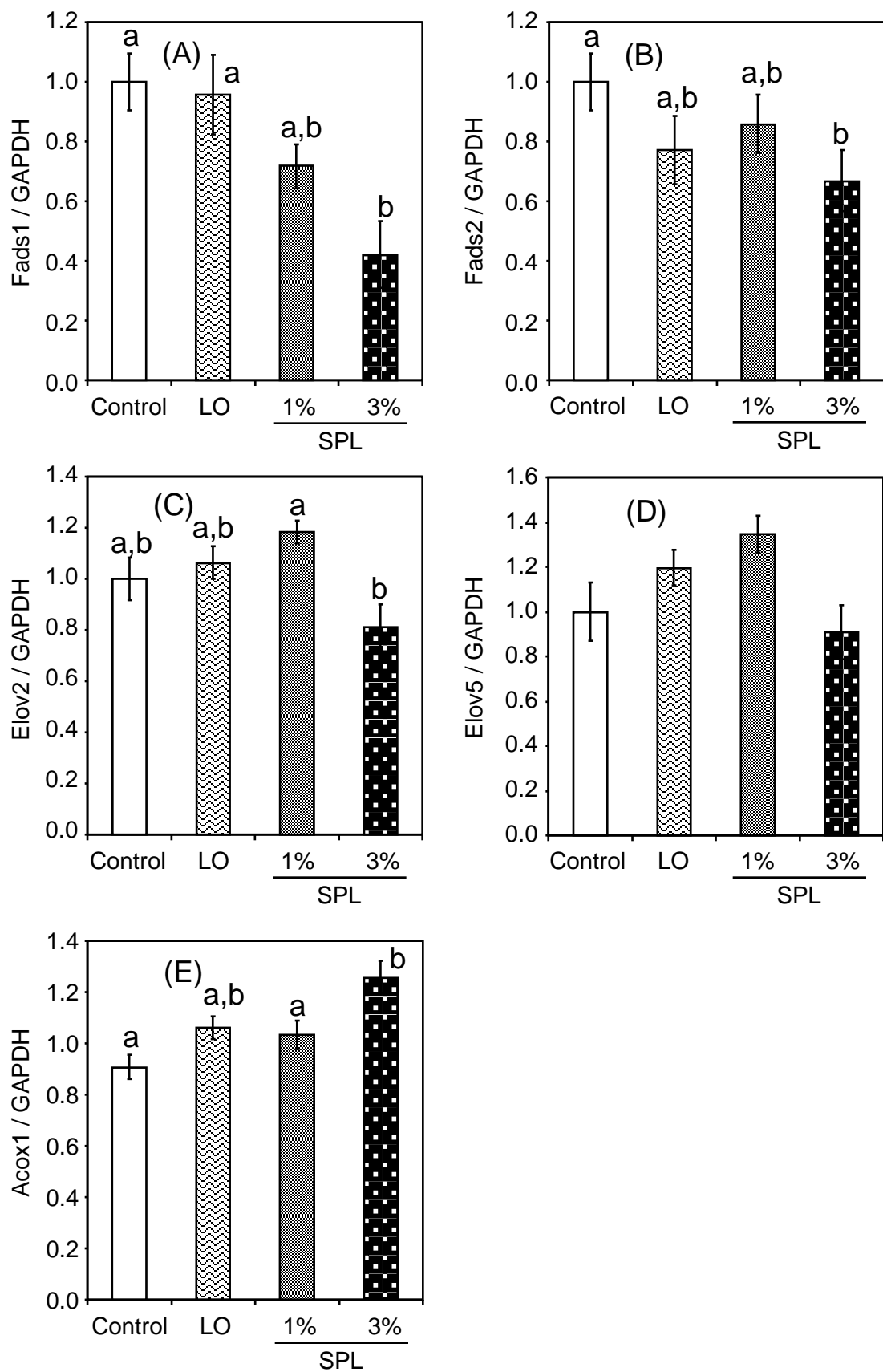


Fig. 2