



Title	Dietary ALA from Spinach Enhances Liver n-3 Fatty Acid Content to Greater Extent than Linseed Oil in Mice Fed Equivalent Amounts of ALA
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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**

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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 α -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, α -linolenic acid absorption, eicosapentaenoic acid,
31 docosapentaenoic acid, arachidonic acid

32
33 **Abbreviations**

34 ALA α -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 α -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 (CH_3ONa) 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 Δ^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 Δ^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 ± 2.14 , 24.13 ± 2.63 , 23.45 ± 2.53 , 23.80 ± 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 Δ^6 -desaturase (Fads2), elongase-5 (Elov5), Δ^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 α -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 (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 ^b	9.79±1.60 ^{a,b}	9.07±0.70 ^a	8.39±1.00 ^a
18:3n-3	0.11±0.06 ^a	1.66±0.38 ^c	0.64±0.09 ^b	1.79±0.27 ^c
20:3n-6	2.08±1.04 ^b	1.30±0.32 ^{a,b}	1.41±0.10 ^{a,b}	1.13±0.12 ^a
20:4n-6	14.16±7.08 ^b	6.51±0.57 ^a	7.80±0.73 ^a	5.68±0.70 ^a
20:5n-3	0.07±0.07 ^a	2.30±0.45 ^c	1.19±0.21 ^b	3.28±0.32 ^d
22:5n-3	0.09±0.10 ^a	0.98±0.27 ^c	0.58±0.12 ^b	1.26±0.15 ^d
22:6n-3	4.29±2.04 ^a	8.62±2.52 ^b	7.02±0.85 ^{a,b}	7.96±1.17 ^b
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 ^b	18.02±2.53 ^a	18.67±1.49 ^a	15.50±1.83 ^a
Total n-3	4.57±2.08 ^a	13.88±2.88 ^c	9.48±1.14 ^b	14.47±1.86 ^c

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 (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 ^b	2.72±0.36 ^{a,b}	2.79±0.88 ^{a,b}	2.36±1.23 ^a
18:3n-3	0.09±0.05 ^a	0.77±0.16 ^{b,c}	0.43±0.13 ^{a,b}	1.04±0.57 ^c
20:4n-6	0.91±0.27 ^b	0.58±0.14 ^{a,b}	0.56±0.26 ^a	0.37±0.19 ^a
20:5n-3	0.00±0.00 ^a	0.17±0.05 ^c	0.08±0.04 ^{a,b}	0.15±0.08 ^{b,c}
22:5n-3	0.02±0.01 ^a	0.14±0.03 ^b	0.08±0.04 ^{a,b}	0.16±0.08 ^b
22:6n-3	0.28±0.11 ^a	0.51±0.10 ^b	0.39±0.16 ^{a,b}	0.35±0.18 ^{a,b}
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 ^b	3.48±0.46 ^{a,b}	3.55±1.17 ^{a,b}	2.85±1.45 ^a
Total n-3	0.38±0.14 ^a	1.59±0.18 ^b	0.98±0.32 ^{a,b}	1.70±0.87 ^b

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 ^b	0.08±0.05 ^b	0.32±0.05 ^a	0.38±0.01 ^{a,b}
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 ^a	0.12±0.12 ^{a,b}	0.05±0.10 ^{a,b}	0.18±0.11 ^b
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 $P < 0.05$.

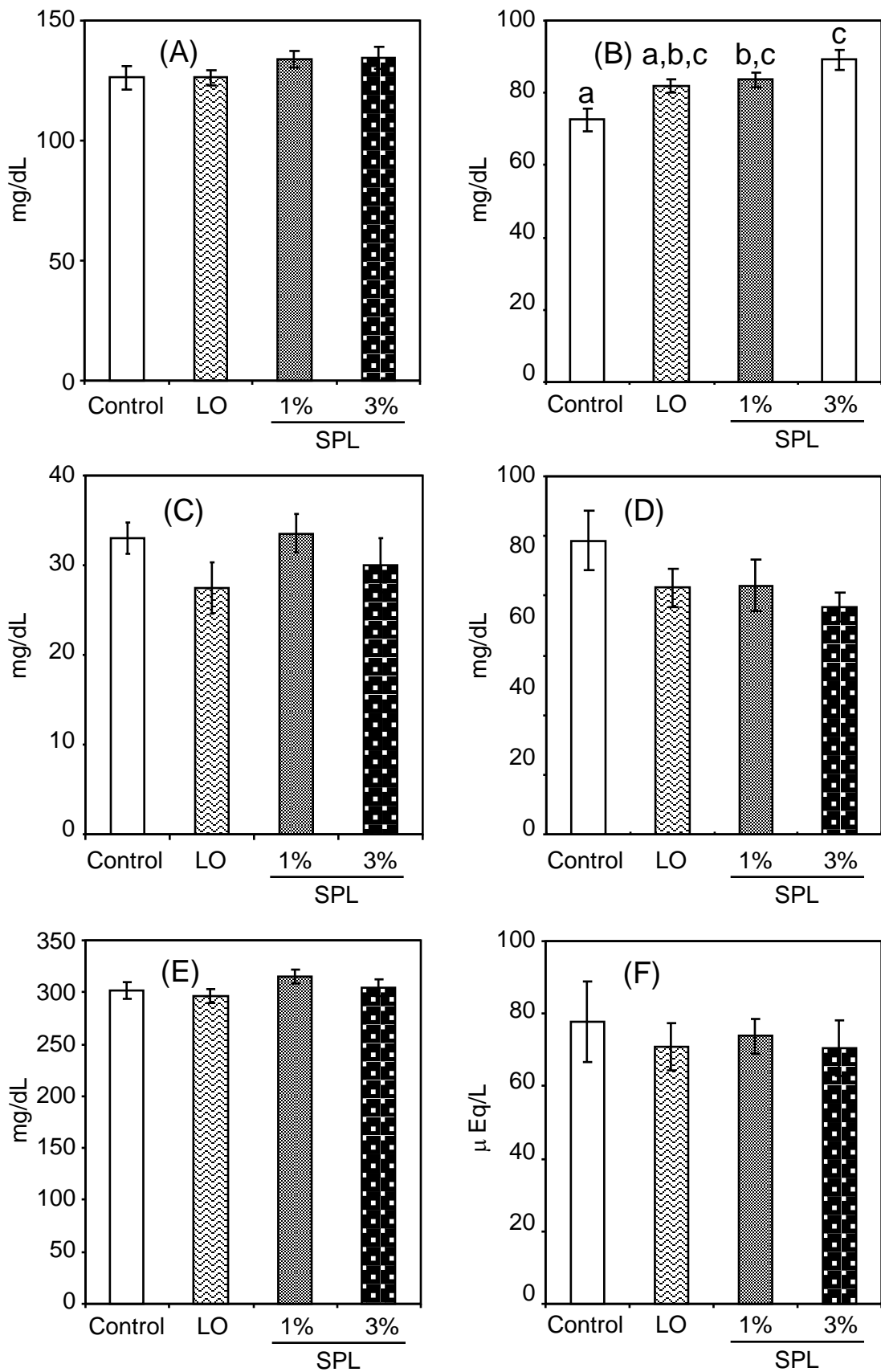


Fig. 1

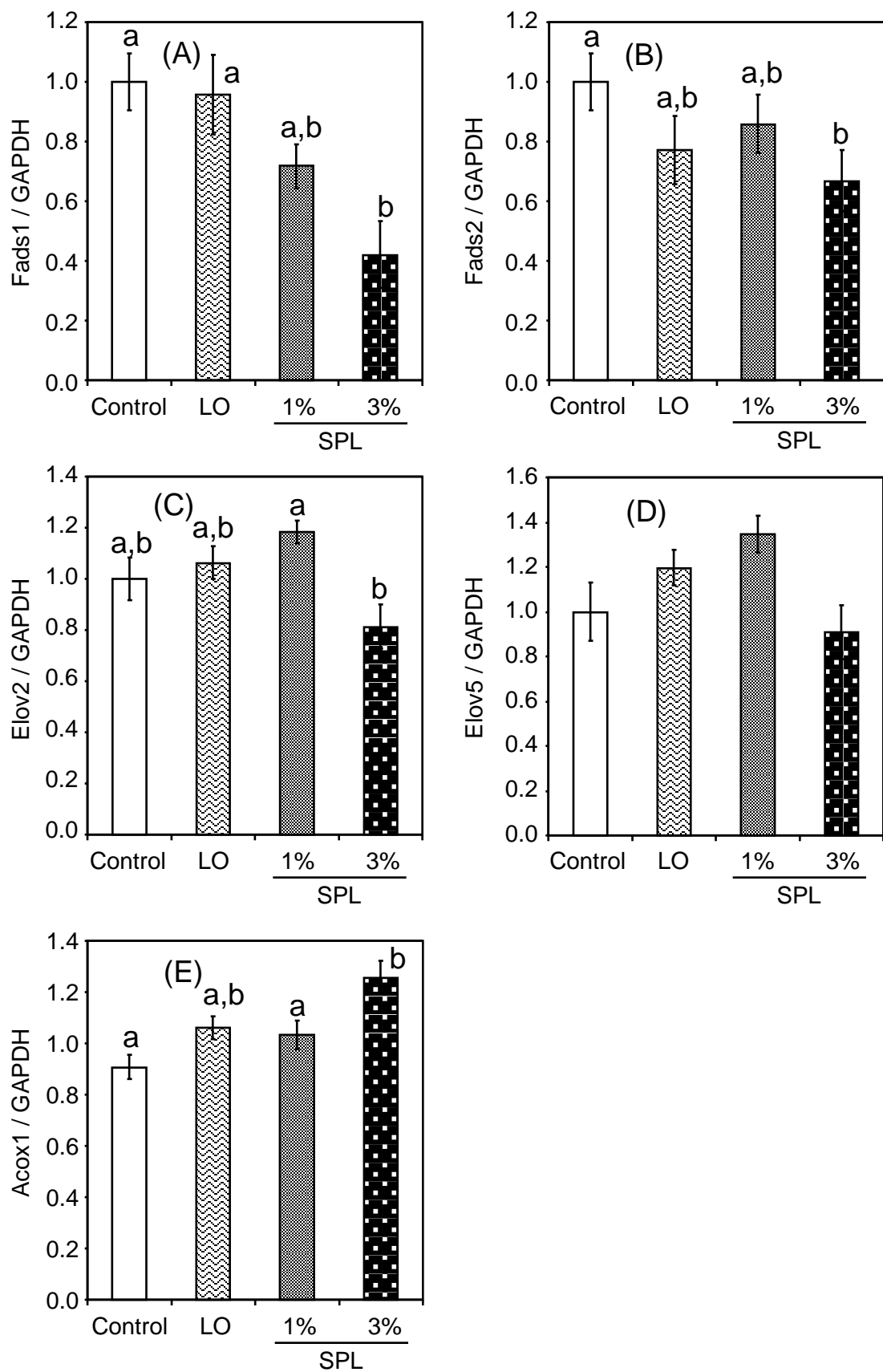


Fig. 2