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GABA-enriched Fermented *Laminaria japonica* Protects against Alcoholic Hepatotoxicity in Sprague-Dawley Rats

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The sea tangle, *Laminaria japonica* has long been used in Korea as a folk remedy to promote health. Gamma-amino butyric acid-enriched (5.56% of dry weight) sea tangle was obtained by fermentation with *Lactobacillus brevis* BJ-20 (FLJ). A suppressive effect of FLJ on carbon tetrachloride-induced hepatotoxicity has been shown previously. Alcohol administration to Sprague-Dawley rats leads to hepatotoxicity, as demonstrated by heightened levels of hepatic marker enzymes as well as increases in both the number and volume of lipid droplets as fatty liver progresses. However, FLJ attenuated alcohol-induced hepatotoxicity and the accumulation of lipid droplets following ethanol administration. Additionally, FLJ increased the activities and transcript levels of major alcohol-metabolizing enzymes, such as alcohol dehydrogenase and aldehyde dehydrogenase, and reduced blood concentrations of alcohol and acetaldehyde. These data suggest that FLJ protects against alcohol-induced hepatotoxicity and that FLJ could be used as an ingredient in functional foods to ameliorate the effects of excessive alcohol consumption.

Key words: Alcohol dehydrogenase, Alcoholic hepatotoxicity, Aldehyde dehydrogenase, Gamma-amino butyric acid, *Laminaria japonica* (sea tangle)

Introduction

The sea tangle, *Laminaria japonica* has long been used as a folk remedy to promote physical health, and is a popular dietary supplement in Korea (Jin et al., 2004). *L. japonica* has recently attracted much attention due to its high dietary fiber, protein, carbohydrates, minerals, and phenolic compound content (Kawano et al., 2007). Recently, anti-oxidant, anti-mutagenic, anti-bacterial, anti-diabetic and anti-obesity properties of *L. japonica* have been documented (Okai et al., 1993; You et al., 2009).

Alcohol is oxidized in the liver by alcohol dehydrogenase (ADH) to the more toxic acetaldehyde, and then to acetate by aldehyde dehydrogenase (ALDH) (Lee et al., 2009). These intermediary metabolites induce the symptoms of hangover, such as thirst, vomiting, fatigue, headache, abdominal pain and lipid peroxidation-mediated cytotoxicity (Morse et al., 2000; Castilla et al., 2004). Recently, diverse natural products have been screened for their capacity to ameliorate such symptoms (Park et al., 2002; Lee et al., 2009; Giriwono et al., 2010). Some of them were found to lower blood alcohol and acetaldehyde levels by enhancing the activities of ADH and ALDH. Previous studies reported that alcohol co-administered with *Lactobacillus brevis* HY7401 or *Lactobacillus* sp. OPK2-59 with high gamma-aminobutyric acid (GABA)-producing activity blocked alcohol absorption in the small intestine, increased the activities of hepatic ADH and ALDH, and thereby reduced considerably blood alcohol and acetaldehyde levels (Ahn et al., 2004; Bae et al., 2009).

GABA, a non-protein amino acid, is produced by the α-decarboxylation of glutamic acid by a

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glutamate decarboxylase (Ueno, 2000). GABA has been reported to mediate several physiological functions involved in neurotransmission, hypotension, tranquilization, and the prevention of diabetic conditions (Inoue et al., 2003; Cho et al., 2007). This has prompted a focus on the development of functional foods containing high GABA levels. GABA is produced by lactic acid bacteria, such as *L. brevis, L. paracasei, L. buchneri,* and *L. sakei* (Yokoyama et al., 2002; Komatsuzaki et al., 2005; Choi et al., 2006; Cho et al., 2007; Kim et al., 2009; Kook et al., 2010). We isolated a GABA-producing *L. brevis* strain BJ20 from *Jotgal*, a traditional Korean fermented food (Lee et al., 2010a). Fermentation of *L. japonica* using *L. brevis* BJ20 results in the complete conversion of glutamic acid into GABA (Lee et al., 2010a). GABA-enriched foods have also been used as a dietary supplement for treatment of sleeplessness, depression, autonomic disorders, chronic alcohol-related symptoms, and hypertension (Inoue et al., 2003; Ahn et al., 2004; Cho et al., 2007; Bae et al., 2009).

We have produced fermented *L. japonica* (FLJ) using *L. brevis* BJ20, and its increased antioxidant capacity was observed with respect to DPPH scavenging, superoxide radical scavenging, and xanthine oxidase inhibition (Lee et al., 2010a). Additionally, it effectively protected rats against carbon tetrachloride-induced hepatotoxicity (Lee et al., 2010b). The objective of this study was to investigate the effect of GABA-enriched FLJ on alcohol-induced hepatotoxicity by assaying hepatic enzyme activities and biochemical markers associated with alcohol metabolism.

Materials and Methods

Preparation of fermented *L. japonica* (FLJ)

L. japonica was added to water at a ratio of 1:15 (w/v) and 2% (w/w of *L. japonica*) rice flour was added to aid fermentation. After autoclaving at 121°C for 30 min, *L. brevis* BJ20 (Accession no. KCTC 11377BP) culture was added to the *L. japonica* solution at a concentration of 2% (v/v), followed by thorough mixing and incubation at 37°C (Lee et al., 2010a). The fermented product was obtained by filtration and was freeze-dried.

GABA analysis

Lyophilized FLJ and non-fermented *L. japonica* were suspended in water and filtered through a 0.2 μm membrane syringe filter (Sartorius Stedim Biotech). The GABA content was then assayed using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Each system consisted of a binary pump, a column oven, a fluorescence detector (FLD), and an autosampler. A Zorbax Eclipse C_{18} column AAA (4.6×150 mm; 3.5 μm; Agilent Technologies) was used for the chromatographic separation. The mobile phases used were A (40 mM Na_2HPO_4; pH 7.8) and B (45% acetonitrile, 45% methanol, 10% water). According to the injector program, each sample was derivatized with *o*-phthaldealdehyde 3-mercaptopropionic acid (OPA-3MPA) (Schwarz et al., 2005). OPA-3MPA-derivatized samples were separated at a column temperature of 40°C and a flow rate of 2.0 mL/min according to the gradient method (Schwarz et al., 2005). GABA derivatized with OPA-3MPA was detected by FLD with excitation at 340 nm, emission at 450 nm, and a PMT gain of 10. GABA content was calculated using a commercial GABA standard (Sigma-Aldrich Co., St. Louis, MO, USA) based on a standard curve. The retention time of the GABA standard was 10.42 min (Fig. 1).

Animal and experimental design

Seven week-old male Sprague Dawley rats were obtained from Hyochang Science Animals Co. (Daegu, Korea). Animals were housed individually in suspended wire-mesh stainless steel cages at room temperature (21-24°C) and with lighting from 08:00 to 20:00. The animals were allowed free access to a commercial diet for 1 week before the experiment. They were then randomly divided into three experimental groups (n=6 each) based on the following dietary regimens: a normal group provided water, an alcohol-fed control group provided an alcoholic beverage containing 30% ethanol (v/v), and an alcohol+FLJ-fed group given alcohol and 5% FLJ (w/w) (Table 1). Ethanol levels were increased gradually, from 10% (v/v) during the first week to 20% (v/v) during the second week. Ethanol (30% v/v) was then provided for the next 3 weeks. Casein in amounts equal to FLJ was administered to the alcohol-only rats. Food consumption and water intake were measured daily and body weight gain was measured once per week.

Animal care procedures followed *The National Institute of Health Guidelines for the Care and Use of Laboratory Animals*. The animal study protocol was approved by the Institutional Animal Care and Use Committee of Dong-A University.

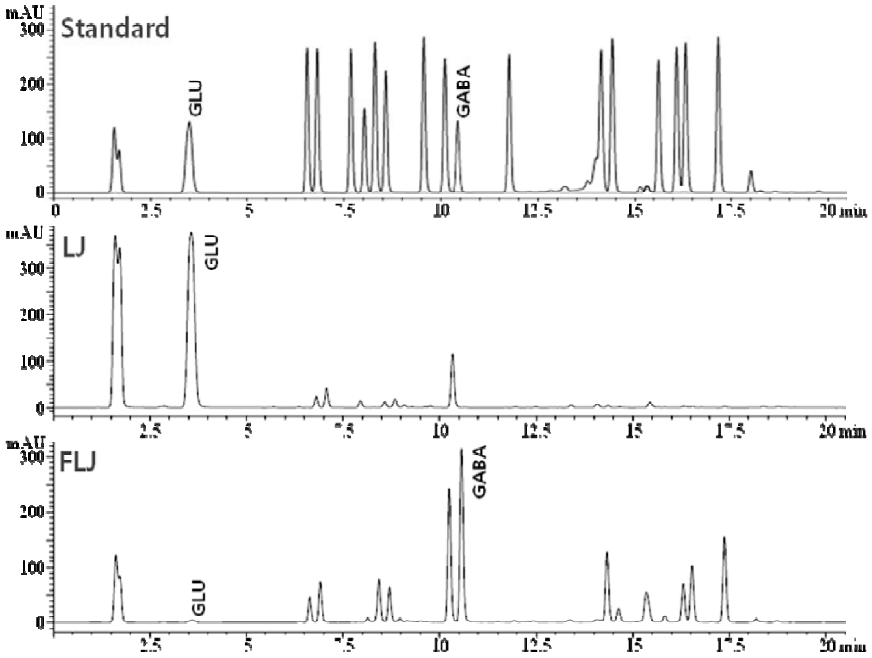

Fig. 1. High-performance liquid chromatography chromatograms of γ-amino butyric acid (GABA) in standard, *Laminaria japonica* (LJ), and fermented *L. japonica* (FLJ). GLU, glutamate.

Table 1. Compositions of experimental diets (%)

Group	Normal	Alcohol	Alcohol + FLJ
Casein	20	20	19
Cornstarch	15	15	12
Sucrose	55	55	55
Cellulose	5	5	4
Corn oil	10	10	10
Mineral mixture*	3.5	3.5	3.5
Vitamin mixture†	1	1	1
Choline bitartrate	0.2	0.2	0.2
DL-Methionine	0.3	0.3	0.3
FLJ	0	0	5

FLJ, Fermented *Laminaria japonica*.
*AIN 93 M-MX mineral mix, MP Biomedicals, Illkirch, France, †AIN 93 VX vitamin mix, MP Biomedicals, Illkirch, France.

Analytical procedure

At the end of the experimental period, rats were sacrificed by withdrawing blood from the abdominal aorta under diethyl ether anesthesia. Serum was obtained by centrifugation (1,026 *g*, 15 min, 4°C). Concentrations of total lipids, triglycerides, and total cholesterol, as well as the activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (γ-GTP), and lactate dehydrogenase (LDH) were measured using a Chemiclinical Chemistry Analyzer from Neodin Medicinal Institute (Seoul, Korea).

Measurement of blood alcohol and acetaldehyde concentrations

Blood alcohol concentration was determined using a commercial UV-test kit (R-Biopharm Co., Ltd., Darmstadt, Germany). This enzymatic test utilized the coenzyme nicotinamide adenine dinucleotide (NAD) with ADH. NADH formation was then measured quantitatively by the increase in absorbance at 378 nm. Blood acetaldehyde concentrations were also measured using a commercial kit that incurporated ALDH.

Determination of ADH and ALDH activities

Liver samples were collected and homogenized in ice-cold 0.25 M sucrose containing 10 mM Tris (pH 7.4), 2 mM dithiothreitol, and 1 mM ethylenediamine tetraacetate using an IKA-ULTRA-TURRAX T25 basic homogenizer (IKA-WERKE GMBH & CO., KG, Staufen, Germany). Protein content was calculated using Bio-Rad protein determination reagents, with bovine serum albumin as the standard. ADH activity was measured using Bergmeyer's method (Bergmeyer, 1974). The conversion of NAD to nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) was monitored by recording changes in absorbance at 340 nm for 5 min after

initiation of the enzyme reaction. ALDH activity was measured using a method described previously (Koivula and Koivusalo, 1975).

Western blot analysis

Hepatic homogenates were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels (20 and 100 μg for ADH and ALDH proteins per lane, respectively), as described previously (Pshezhetsky et al., 1993). Separated proteins were then transferred electrophoretically to a nitrocellulose membrane by the method of Towbin (Towbin et al., 1979). β-Actin was used as a control for protein loading. Proteins on the nitrocellulose membranes were detected using a SuperSignal West Pico Chemiluminescent substrate.

Histology

Liver tissue was fixed in 10% neutral buffered formalin and processed for histological examination according to a standard method and then stained with hematoxylin and eosin.

Statistical analysis

Data are expressed as means ± SD, and all statistical comparisons were made using a one-way analysis of variance (ANOVA), followed by Duncan's test. A *P*-value of <0.05 was considered to indicate statistical significance.

Results and Discussion

Preparation of FLJ and determination of GABA content

GABA is biosynthesized by animals, plants, and microorganisms via the α-decarboxylation of glutamic acid by a glutamate decarboxylase (Ueno, 2000). GABA is produced by most lactic acid bacteria, including *L. brevis, L. paracasei, L. buchneri,* and *L. sakei* B2-16 (Yokoyama et al., 2002; Komatsuzaki et al., 2005; Choi et al., 2006; Kim et al., 2009; Kook et al., 2010). Glutamate is a major substrate for GABA production and is especially abundant in foods such as sea tangle, cheese, soybeans, rice bran, and mushrooms (Park and Oh 2006; Kook et al., 2010; Lee et al., 2010a). In our previous study, GABA-producing *L. brevis* BJ20 was isolated from *Jotgal*, a Korean traditional fermented food. This strain is capable of complete conversion of *L. japonica* glutamic acid into GABA (Lee et al., 2010a). GABA-enriched FLJ has demonstrated a number of biological effects such as hepatotoxicity improvement and anti-oxidant activity (Lee et al., 2010a; 2010b). Thus, FLJ may be a good material for the development of functional health foods.

FLJ was produced according to our previous method (Lee et al., 2010a). The glutamic acid and GABA content are summarized in Table 2. *L. japonica* contained 6.29% (w/w) glutamic acid, but no GABA was detected. However, after fermentation with *L. brevis* BJ20, the GABA content of FLJ was 5.56% (w/w dry weight); thus most of the glutamic acid had been converted to GABA. These results are in agreement with our previous report (Lee et al., 2010a). Additionally, the alanine, valine, glycine, and leucine contents had increased dramatically after fermentation (data not shown).

Table 2. Contents of glutamic acid and γ-amino-butyric acid in *Laminaria japonica*

Free amino acid	*L. japonica*	Fermented *L. japonica*
Glutamic acid (%)	6.29	0.08
γ-Aminobutyric acid (GABA) (%)	0.0	5.56

Effect of the FLJ on hepatic injuries

GABA and/or GABA-enriched foods are used as dietary supplements to help treat chronic alcohol-related symptoms, sleeplessness, and depression, and improve hypertension (Inoue et al., 2003; Cho et al., 2007). Additionally, our previous results revealed that GABA-enriched FLJ has high anti-oxidant activities with regard to DPPH scavenging, superoxide radical scavenging, and xanthine oxidase inhibition (Lee et al., 2010a). We thus determined whether GABA-enriched FLJ could suppress alcohol-induced hepatotoxicity in rats.

Table 3 summarizes body weight gain, food intake, water intake, and relative tissues weights of rats during the experimental period. The animals in the alcohol-fed group had significantly decreased final body weight gains, food intakes, and water intakes. However, the alcohol+FLJ-fed group had slightly increased final body weight gain, food intake, and water intake compared to the alcohol-fed group. The relative tissue weights of the liver, testis, kidneys, spleen, and heart, and all groups showed similar tissue weights.

Serum levels of hepatic enzymes, such as ALT, AST, γ-GTP, and LDH, are used as biochemical markers of hepatic injury (Kojima et al., 2005; Cha et al., 2009). These enzymes can be present at high concentrations when the liver has been damaged by excessive alcohol intake. While their ratios may vary, liver damage due to chronic ethanol consumption has been indicated by a ratio of AST to ALT of greater than 2 (Kojima et al., 2005). In our study, liver injury

Table 3. Effects of fermented *Laminaria japonica* (FLJ) on the body weight, food intake, water intake, and relative tissues weight of alcohol-induced hepatic damaged rats

Group	Normal*	Alcohol†	Alcohol + FLJ‡
Final body weight gain (g)	211.23 ± 9.17^{a}	98.78 ± 21.02^{b}	127.28 ± 34.09^{ab}
Food intake (g/day)	19.80 ± 0.20^{a}	13.40 ± 0.60^{b}	14.33 ± 2.19^{b}
Water intake (mL/day)	35.60 ± 1.47^{a}	14.60 ± 0.81^{b}	19.33 ± 2.85^{c}
Relative tissues weights (%; g/100 g BW)			
Liver	2.67 ± 0.03^{NS}	2.66 ± 0.07	2.75 ± 0.10
Testis	0.76 ± 0.03^{NS}	0.88 ± 0.07	0.83 ± 0.06
Kidney	0.77 ± 0.04^{NS}	0.85 ± 0.04	0.83 ± 0.08
Spleen	0.18 ± 0.01^{NS}	0.18 ± 0.01	0.17 ± 0.02
Heart	0.84 ± 0.47^{NS}	0.39 ± 0.01	0.42 ± 0.03

Rats were fed with ethanol or FLJ as described in the materials and methods. Values with different letters are significantly different at $P<0.05$ (mean±SD, $n=6$).
NS, not significant different.
*Provide water, †Provide an alcoholic beverage containing 30% ethanol (v/v), ‡Provide up to 30% ethanol (V/V) and 5% FLJ (w/w).

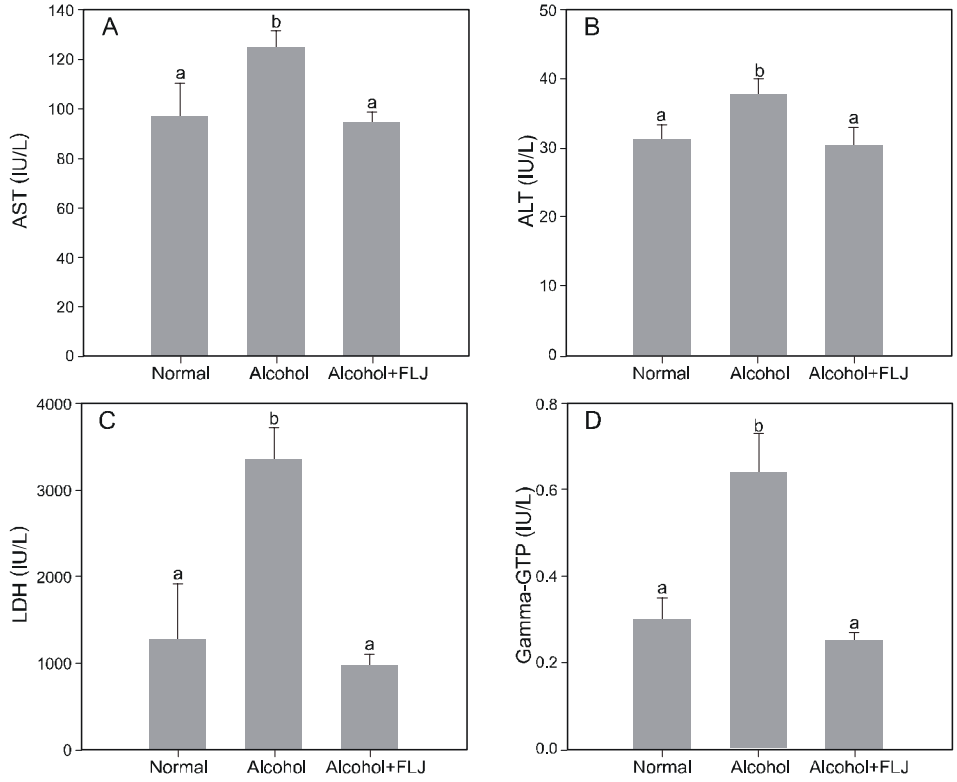

Fig. 2. Effects of fermented *Laminaria japonica* (FLJ) on the serum activities of (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) lactate dehyfrogenase (LDH), and (D) γ-glutamyl transpeptidase (γ-GTP) in alcohol-induced hepatic damaged rats. Values with different letters are significantly different at $P<0.05$ (mean±SD, $n=6$).

was sustained in individual rats due to chronic ethanol treatment, as indicated by high concentrations of hepatic enzymes in the serum. However, the alcohol+FLJ-fed group showed effective suppression of injury, as evidenced by decreased serum ALT and AST, γ-GTP, and LDH activities, similar to those in the normal group (Fig. 2). Liver marker enzymes such as ALT, AST, γ-GTP, and LDH are cytoplasmic in nature; upon liver injury, these enzymes enter into the circulatory system due to altered membrane

permeability (Arun and Asha, 2007). Thus, concentrations of ALT, AST, γ-GTP, and LDH will increase in the serum of rats fed ethanol. We demonstrated that GABA-enriched FLJ decreased serum levels of ALT, AST, γ-GTP, and LDH. Recently, several studies have demonstrated that supplementation with GABA-producing *Lactobacillus* sp. OPK2-59 powder, GABA plus carnitine, and fermented barley extract attenuated chronic alcohol-induced liver damage, as shown by decreased levels of ALT and AST (Soh et al., 2003; Giriwono et al., 2010). Our previous data also indicate that GABA-enriched FLJ possesses significant antioxidant activities, and may thus reduce levels of ALT, AST, γ-GTP, and LDH levels (Lee et al., 2010b). Indeed, FLJ contains a high concentration of GABA (5.56%), a known antioxidant (Table 2).

Histological observations were largely in agreement with the serum enzyme levels. Alcohol treatment induced a marked accumulation of lipid droplets in hepatocytes (Fig. 3). However, rats that received alcohol+FLJ had lower levels of lipid droplets in hepatocytes. Normal rats revealed clear-cut hepatic lobules with a uniform pattern of polyhedral hepatocytes radiating towards the periphery from the central vein.

Table 4 shows the effects of FLJ supplementation on serum lipid concentrations in alcohol-fed rats. Liver steatosis is related to chronic ethanol consumption and higher hepatic lipid concentrations cause liver injury. Serum total lipid concentrations showed a tendency to decrease slightly in the alcohol- and alcohol+FLJ-fed compared with the normal group. However, triglyceride and free fatty acid levels were significantly higher in the alcohol-fed, but not the alcohol+FLJ-fed, group. Alcohol intake significantly increases serum triglyceride levels, resulting in hypertriglyceridemia (Park et al., 2002; Cha et al., 2009; Giriwono et al., 2010), and increased serum triglycerides have been reduced by supplementation with GABA-producing *Lactobacillus* sp. OPK2-59 powder, zinc-enriched yeast, and GABA (Park et al., 2002; Soh et al., 2003; Bae et al., 2009; Giriwono et al., 2010). Total serum cholesterol levels were significantly decreased in both alcohol and alcohol+FLJ-fed groups compared with the normal group.

Effect of the FLJ on alcoholic hepatotoxicity-related enzymes

Alcohol is oxidized to acetaldehyde by ADH primarily in the liver and further metabolized to acetate by ALDH (Höög et al., 2001). Acetaldehyde is more toxic than alcohol and thus exerts cytotoxicity at lower concentrations (Signorini-Allibe et al., 2005). Acetaldehyde causes hangover symptoms, such as thirst, vomiting, fatigue, headache, and abdominal pain as well as causing cytotoxicity and fatty liver (Signorinoi-Allibe et al., 2005). Recently, many natural products have been screened for their capacity to deviate from a hangover and fatty liver (Park et al., 2002; Lee et al., 2009; Giriwono et al., 2010). Some were found to lower blood alcohol and acetaldehyde levels by enhancing the activities of alcohol-metabolizing enzymes (Cha et al., 2009; Lee et al., 2009). Indeed, administration of *L. brevis* HY7401 or GABA-producing *Lactobacillus* sp. OPK2-59 powder blocked alcohol absorption in the small intestine and increased hepatic ADH and

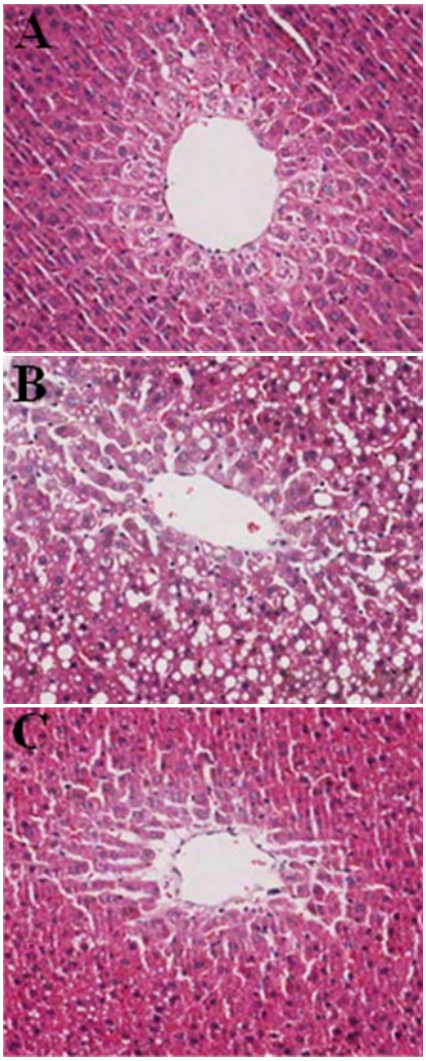

Fig. 3. Hepatic histopathologic changes in normal (A), alcohol-fed control (B), and alcohol+fermented *Laminaria japonica* (FLJ) feeding groups (C) (×200).

Table 4. Effects of fermented *Laminaria japonica* (FLJ) on serum lipid concentrations in alcohol-induced hepatic damaged rats

Group	Normal	Alcohol	Alcohol + FLJ
Total lipid (mg/dL)	262.60 ± 20.71[a]	221.20 ± 8.07[ab]	192.33 ± 21.67[b]
Triglyceride (mg/dL)	47.80 ± 3.90[b]	58.60 ± 4.95[a]	40.33 ± 1.86[b]
Cholesterol (mg/dL)	64.40 ± 1.66[a]	56.00 ± 2.83[b]	48.00 ± 2.65[c]
Free fatty acid (mmol/L)	0.83 ± 0.11[a]	1.17 ± 0.14[b]	0.65 ± 0.04[a]

Rats were fed with ethanol or FLJ as described in Materials and Methods. Values with different letters are significantly different at $P<0.05$ (mean±SD, $n=6$).
*Provide water, †Provide an alcoholic beverage containing 30% ethanol (v/v), ‡Provide up to 30% ethanol (V/V) and 5% FLJ (w/w).

ALDH activities in the liver, and thereby reduced the blood alcohol and acetaldehyde levels (Ahn et al., 2004; Bae et al., 2009).

Alcohol was not detected in the blood of rats fed a normal diet (Fig. 4); however, the blood alcohol concentration of the alcohol-fed group was 0.043%. FLJ administration decreased the blood alcohol concentration to 0.031%. Additionally, the rats in the alcohol+FLJ-fed group showed decreased acetaldehyde concentrations compared with those in the alcohol-fed group. These lower blood alcohol and acetaldehyde levels might be due to modulation of hepatic alcohol-metabolizing enzymes. Thus, we also investigated the effects of GABA-enriched FLJ on ADH and ALDH activities. The alcohol-fed group had an ADH activity similar to that of the normal group; however, the alcohol+FLJ-fed group had significantly augmented ADH activity compared with the alcohol-fed group (Fig. 5). Additionally, the alcohol+FLJ-fed group had significantly increased ALDH activity compared with both the normal and alcohol-fed groups. Levels of ADH and ALDH transcripts were markedly increased in the alcohol+FLJ-fed group again compared with both the normal and alcohol-fed groups (Fig. 6). Thus, the hepatic ADH and ALDH activities, transcript levels, and blood alcohol and acetaldehyde levels were well correlated. These data suggest that administration of GABA-enriched FLJ or lactic acid bacteria may rapidly mitigate ethanol-induced hepatic damage by increasing ADH and ALDH activity. Additionally, feeding of *Lactobacillus* sp. GG to rats has been demonstrated to reduce both endotoxemia and the severity of alcohol-induced experimental liver injury in chronically ethanol-fed rats (Nanji et al., 1994).

In conclusion, this study demonstrated that supplementation with GABA-enriched FLJ decreased both blood alcohol and acetaldehyde levels by

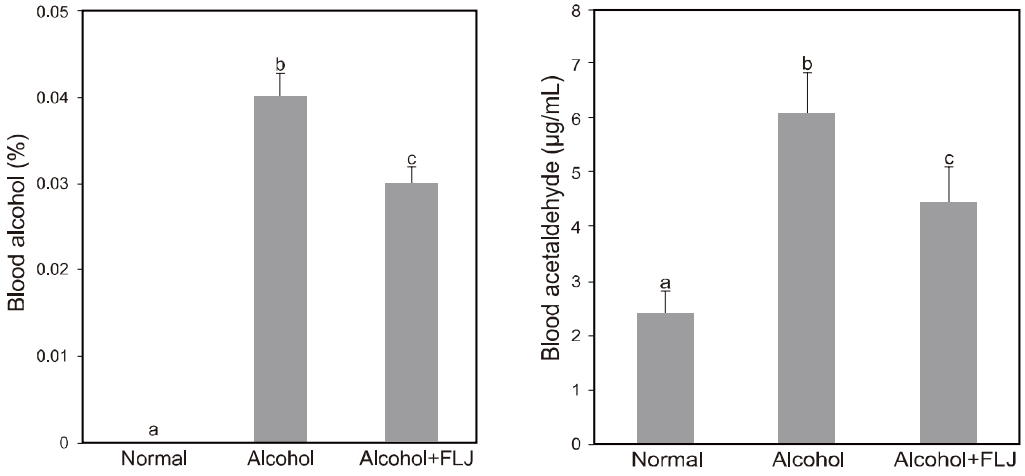

Fig. 4. Effects of fermented *Laminaria japonica* (FLJ) on the contents of alcohol and acetaldehyde in alcohol-induced hepatic damaged rats. Values with different letters are significantly different at $P<0.05$ (mean±SD, $n=6$).

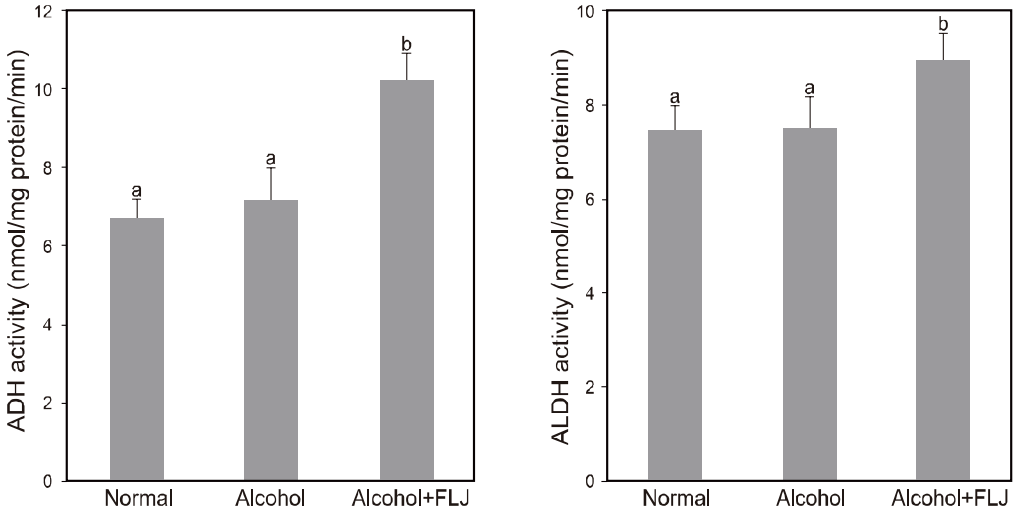

Fig. 5. Effects of fermented *Laminaria japonica* (FLJ) on the hepatic activities of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in alcohol-induced hepatic damaged rats. Values with different letters are significantly different at $P<0.05$ (mean±SD, $n=6$).

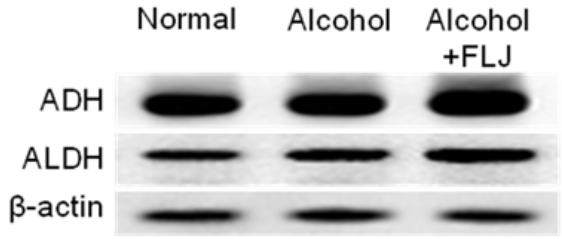

Fig. 6. Effects of fermented *Laminaria japonica* (FLJ) on the hepatic alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) mRNA expressions in alcohol-induced hepatic damaged rats.

modulating the expression and activity of alcohol metabolizing enzymes (including ADH and ALDH) and thus prevented alcoholic liver damage. The findings of this study suggest that GABA-enriched FLJ may be useful for ameliorating the symptoms of alcoholic hangover and ethanol-induced hepatocyte toxicity. However, determination of the precise mechanism of reduction of alcohol-induced hepatic toxicity by GABA-enriched FLJ is now necessary.

Acknowledgments

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