Green Tea Extract (Camellia sinensis) Fermented by Lactobacillus fermentum Attenuates Alcohol-Induced Liver Damage

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Here, the impact of an extract derived from green tea (Camellia sinensis) and fermentation with Lactobacilli fermentum strain OCS19 was explored with acute alcohol-induced liver damage. The study employed the HepG2 hepatic cell line and an in vivo murine model of liver damage. L. fermentum-fermented green tea extract (FGTE) was found to possess pronounced alcohol metabolizing enzyme activity. It significantly enhanced the cell viability of HepG2 cells following of them exposure, to ethanol (p < 0.05) as compared with an extract derived from Hovenia dulcis, a positive control that is known for its action as an alcohol antagonist. Our in vivo studies indicated that prior administration of FGTE to alcohol-exposed mice significantly prevented subsequent increases in blood alcohol concentration (p < 0.05), in addition to the induction of serum alanine aminotransferase (ALT) and triglycerides (p < 0.05). Furthermore, the activity of hepatic alcohol dehydrogenase (ADH) and its mRNA expression level both increased in the livers of mice treated with FGTE, similarly to the H. dulcis-treated group. Taken together, these results may suggest that green tea extract coupled with L. fermentum fermentation attenuates the risk of ethanol-induced liver damage.

Key words: alcohol-induced liver damage; fermented green tea extract; Lactobacillus fermentum

Green tea (Camellia sinensis) is employed to make one of the most popular beverages in the world. Cathechins (flavan-3-ols, or tea polyphenols) are major components of green tea, and have been reported to have various physiological properties, including antioxidant, antiallergic, antimutagenic/anticarcinogenic, and antibacterial.1) Of importance to the current study, some reports have confirmed a hepatoprotective role of green tea against ethanol intoxication.2–4)

Probiotics such as lactic acid bacteria (LAB) are defined as living microorganisms which, when administered in adequate amounts, confer a health benefit upon the host.5) Probiotics have been linked to various biological actions, including stimulation of the immune system, balancing of intestinal microbiota, potential reduction of inflammation, and the prevention of allergies, hypertension, and cancer.6,7) It has also been reported that probiotics can ameliorate due to hepatotoxicity.7–9) Moreover, certain species of lactobacilli have been found to lower blood alcohol and acetaldehyde levels in animals exposed to by enhancing the activities of alcohol-metabolizing enzymes.10,11)

Alcohol, in moderation, can promote metabolism in the body and help to reduce the risk of heart disease, but, heavy drinking can cause so-called hangovers and other health impairments. Excessive intake of alcohol can damage organs such as the liver and heart, resulting in dysfunction reflected by elevated triglyceride levels.12) Alcohol is rapidly oxidized to acetaldehyde by alcohol dehydrogenase (ADH), cytochrome P-450 type 2E1, and catalase, and then is further metabolized to acetic acid by aldehyde dehydrogenase (ALDH). The ADH pathway is therefore a major pathway of alcohol metabolism. There is considerable polymorphism in the ADH pathway, which is apparently related both to the production of acetaldehyde and to first-pass elimination of alcohol.13)

The development of new materials that can (i) enhance the activities of hepatic and gastric ADH and ALDH and (ii) promote hepatoprotective effects to prevent ethanol-induced damage is a leading strategy for the improvement of liver injury caused by alcohol.14,15) The goal of the present study was to determine the impact of certain probiotics, green tea extract (GTE) and its fermented product with Lactobacillus fermentum strain OCS19, on alcohol-induced toxicity and liver injury, both in vitro and in vivo.

Materials and Methods

Chemical and reagents. Dimethyl sulfoxide (DMSO), 3’-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and nicotinamide adenine dinucleotide (NAD) were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were from HyClone Laboratories (Logan, UT). An EnzyChrom™ ethanol assay kit (ECET-100) was from BioAssay Systems (Hayward, CA). TRIZOL™ reagent, Superscript transcriptase, and a Superscript III RT-PCR kit were from Invitrogen (Carlsbad, CA). SYBR green PCR Master Mix was from Bio-Rad (Hercules, CA).

Sample preparation. A total of seven strains of Lactobacillus (Table 2) were used. They were obtained from the Food Microbiology Laboratory of Korea University (Seoul, Korea). The strains were of various origins, including the human gastrointestinal (GI) tract, infant feces, and the intestinal contents of pigs. The strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Difco, MD) at 37°C for

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18 h. They were sub-cultured 3 times prior to use to maintain their activities. For enzyme assays, mature Lactobacillus cells were washed with phosphate buffered saline (PBS) and sonicated 5 times for 30 s per time in an ice bath. The supernatants were centrifuged at 100,000 x g for 60 min.

Fresh *Camellia sinensis* (green tea) and *Hovenia dulcis* Thunb. were purchased from a local herb market (Kyung-Dong Market, Seoul, Korea). Green tea leaves and *H. dulcis* were dried and ground using a blender. Each sample (150 g) was boiled with 1.5 L of water for 3 h, and then cooled to room temperature. The supernatants were passed through Whatman filter paper no. 2 (Maidstone, UK). All the filtrates were evaporated and freeze-dried in a lyophilizer (Ishin, Seoul, Korea). For the production of fermented green tea extract (FGTE), *L. fermentum* OCS19 was cultured in MRS medium with the addition of green tea extract (GTE, 10 mg/mL) at 37 °C for 24 h.

Extraction and analysis of crude catechins in GTE and FGTE. The crude catechin contents of GTE and FGTE were determined by the spectrophotometric method of Li et al. Briefly, each sample (approximately 0.1 g) was placed into a 100-ml flask, followed by the addition of 80% ethanol (30 mL). Next, each sample was heated in a water bath for 15 min at 60 °C, sonicated for 5 min, and then filtered through a 0.45-μm Millipore nylon filter. For crude catechin analysis, the filtrate (5 mL) was transferred into a 50-ml volumetric flask and then reacted with 5 mL of dyeing solution (0.1 g of ferrous sulfate and 0.5 g of potassium sodium tartrate tetrahydrate, dissolved in 100 mL of distilled water) and 15 mL of buffer (0.01 M potassium phosphate, pH 7.5). After several minutes for color development, the absorbance was measured at 540 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA). A blank solution was prepared, distilled water replacing the tea extract.

Cell viability assay. Cell viability was assessed by MTT assay. HepG2 human hepatoma cells (1 x 10^5 cell/well; Korea Cell Line Bank, Seoul, Korea) were seeded in triplicate wells of a 96-well microplate and cultured in RPMI medium (200 μL) for 24 h. Next the cells were pretreated for 24 h with vehicle control (PBS) or a filtrate of *L. fermentum* adjusted to pH 6.5, *H. dulcis* extract (hereafter referred to simply as *H. dulcis*, 2.5 mg/mL), GTE (1.25 mg/mL), or FGTE (1.25 mg green tea/mL), followed by treatment with 0.4 M ethanol or 25 mM acetaldehyde for 24 h. Then MTT solution (100 μL, 2 mg/mL in PBS) was added to each well. After 4 h of incubation, the solution was discarded and the cells were solubilized with 100 μL of DMSO for 30 min. The MTT signal intensity was determined spectrophotometrically by measuring the absorbance at 570 nm using an ELISA reader (Molecular Devices). Cell viability was assessed by comparing the MTT fluorescence signal intensity of the treated cells with the signal intensity of the control cells and expressing the result as a percentage of control.

Animal administrations. Six-week-old male Imprinting Control Region (ICR) mice were purchased from Samtako (Osan, Korea). They were acclimatized for 1 week before the experiment, and were housed in plastic cages in a room maintained at 22 °C under a 12 h day/night cycle throughout the experiment. The mice were divided into six groups (n = 9 for each group) based on sample treatment, as follows: (i) normal (untreated) group; (ii) water-pretreated group; (iii) *L. fermentum* (10^7 CFU/kg)-pretreated group; (iv) GTE (50 mg/kg)-pretreated group; (v) FGTE (50 mg of green tea/kg + 10^7 CFU/kg *L. fermentum*) -pretreated group; and (vi) *H. dulcis* (41 mg/kg)-pretreated group. The Korea Food and Drug Administration (KFDA) gave approval in 2008 for the use of extracts of *H. dulcis* for protection and aid in recovery of the liver from substances such as alcohol. The effective dose of *H. dulcis* is 2,460 mg/d.1^{17} Hence, the mice were administered *H. dulcis* extract according to this dosage (2,460 mg/60 kg of body weight/d). In addition, GTE and FGTE were administered at doses of 900 mg of catechin/60 kg of body weight/d. GTE is effective at this concentration against oxidative stress and has been approved for such use by the KFDA at 300–1,000 mg of catechin/d.1^{19} Feedings were done orally by gavage once daily for 7 d. Thirty min after the final treatment, the mice in groups (ii) through (vi) received an acute ethanol dose of 3 g/kg of body weight diluted in water. The control group mice received an isocaloric maltose solution (5.5 g/kg of body weight) rather than ethanol.

To assess hepatic ADH and ALDH activities and mRNA expression levels, additional mice were sacrificed 1 h after ethanol challenge. The livers were collected and frozen in liquid nitrogen, and stored at −80 °C for further study.

Alcohol metabolism enzyme assay. ADH activity was measured in mature Lactobacillus cells by a modification of the method of Noskova et al. The ADH protein content in the cytosol of each strain was then measured by the method of Bradford et al. and ADH activity was defined as the amount of NADH produced pmol of protein per min. In addition, ALDH activity was measured spectrophotometrically, as for the ADH assay.

Analysis of serum blood alcohol concentration and biochemical factors. Whole blood was collected from the ophthalmic vein and from the experimental mice in a serum separation tube at 1, 3, and 6 h after the ethanol challenge. Then, blood samples were centrifuged at 3,000 rpm for 10 min. The supernatant consisted of blood serum, and was used in the analysis of blood alcohol levels. For this assay, ethanol was quantified using an EnzyChrom™ ethanol assay kit (ECET-100, BioAssay Systems, CA, USA) following to the manufacturer’s instructions.

To analyze the presence and levels of biochemical factors, the animals were sacrificed 6 h after the initial administration of ethanol, and blood was collected by cardiac puncture. Serum was collected as above. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured on a Modular Analytics automatic analyzer (Roche Diagnostics, Mannheim, Germany) by standard methods. Serum triglycerides and total cholesterol were measured enzymatically using the Modular Analytics automatic analyzer.

Histological analysis. A histological examination was performed on the liver samples collected from each animal. The samples were fixed in 10% buffered formalin, dehydrated in ethanol, and then embedded in paraffin. Five-micron-thick liver sections were prepared and stained with hematoxylin and eosin (H&E), and examined under a light microscope.

mRNA expression of alcohol metabolizing enzyme encoding genes. Total RNA was extracted from murine liver tissue, and the total cellular RNA was isolated using TRIZOL™ reagent (Invitrogen, CA, USA) following the manufacturer’s instructions, and further purified using of an acidic phenol/chloroform extraction. cDNA was then generated using Superscript transcriptase III and a Superscript III RT-PCR kit (both Invitrogen). After cDNA synthesis, quantitative real-time PCR was performed using the primers described in Table 1 and an iCycler iQ (Bio-Rad) coupled with SYBR green PCR Master Mix.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference</th>
<th>Sequences</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH1</td>
<td>NM,019286.2</td>
<td>5'-GGGTTGACCTTGTGTAACACC-3'</td>
<td>200bp</td>
</tr>
<tr>
<td>ALDH2</td>
<td>NM,032416.1</td>
<td>5'-CTCGTGGCAGTGAAGTGTTG-3'</td>
<td>226bp</td>
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<tr>
<td>GAPDH</td>
<td>NM,002046.3</td>
<td>5'-ATGACAGTCGTCATGCCATC-3'</td>
<td>271bp</td>
</tr>
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Table 1. Primer Sequences for Real-Time PCR
Results and Discussion

**ADH and ALDH activity of the Lactobacillus strains**

Among the seven Lactobacillus strains studied here, *L. rhamnosus* GG and *L. fermentum* OCS19 demonstrated the highest ADH activity, as shown in Table 2. Of particular relevance to this study, *L. fermentum* OCS19 yielded an ADH activity of 42.93 ± 1.68 unit/mg of protein. The other bacteria showed relatively low ADH activity. Furthermore, OCS19 showed the highest ALDH activity relative to the other strains. These results are important because, during normal alcohol metabolism, some of the absorbed ethanol is oxidized to acetaldehyde in the colon. Accordingly, after alcohol intoxication, some of the absorbed ethanol is oxidized to acetaldehyde in the colon. Consequently, after alcohol administration, the highest acetaldehyde levels in the body are found in the large intestine, and many gastrointestinal bacteria reveal marked levels of ADH and ALDH. Thus, our results might suggest that *L. fermentum* strain OCS19 plays an especially important role in ethanol metabolism in the colon.

**Hepatoprotective effects of Lactobacillus and plant extracts on HepG2 cells**

To determine the protective impacts of Lactobacillus strains and the plant extracts on HepG2 cells, cell viability was investigated by MTT assay. A dose-response study was conducted to determine the concentrations of the samples (*L. fermentum* supernatant adjusted to pH 6.5, GTE at 1.25 mg/mL, FGTE 1.25 mg green tea/mL, and *H. dulcis* 2.5 mg/mL), that presented no cytotoxicity toward the hepatocytes (data not shown). Next, the protective effects of *L. fermentum* OCS19, GTE, FGTE and *H. dulcis* pre-treatment against alcohol were investigated for HepG2 cells subsequently exposed to 0.4 mM ethanol. As expected, the cell viability of the PBS-pretreated, ethanol-treated control group was only 52.2% of the cells not treated with alcohol (Fig. 1A). Cell viability increased significantly, to 74.8–84.0%, for the ethanol-treated cells that were pretreated with *L. fermentum* OCS19, GTE, FGTE or *H. dulcis* (Fig. 1A). In addition, *L. fermentum* and FGTE showed protective effects as compared to PBS against 25 mM acetaldehyde treatment (Fig. 1B). These results indicate that *L. fermentum* and FGTE attenuated cytotoxicity in the ethanol- and acetaldehyde-treated HepG2 cells.

Various mechanisms of action have been reported that might be responsible for the protective actions of substances such as *L. fermentum* and FGTE against alcohol toxicity. These include inhibition of ethanol metabolism, degradation of ethanol and acetaldehyde,

### Table 2. Alcohol-Metabolizing Enzyme Activities of Lactobacillus Strains

<table>
<thead>
<tr>
<th>Lactobacillus strain</th>
<th>Origin</th>
<th>ADH activity (nmol NADH/ min/mg protein)</th>
<th>ALDH activity (nmol NADH/ min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td>Human GI tract</td>
<td>36.84 ± 3.58ab</td>
<td>10.47 ± 0.87ab</td>
</tr>
<tr>
<td><em>L. acidophilus</em> GP1B</td>
<td>Pig intestine</td>
<td>28.43 ± 4.23bc</td>
<td>9.98 ± 0.36bc</td>
</tr>
<tr>
<td><em>L. salivarius</em> E4191</td>
<td>Egyptian infant feces</td>
<td>22.95 ± 1.05cd</td>
<td>8.65 ± 0.66cd</td>
</tr>
<tr>
<td><em>L. gasseri</em> OCS12</td>
<td>Korean infant feces</td>
<td>24.75 ± 3.05cd</td>
<td>6.54 ± 1.07cd</td>
</tr>
<tr>
<td><em>L. fermentum</em> OCS19</td>
<td>Korean infant feces</td>
<td>42.93 ± 1.68bc</td>
<td>11.30 ± 1.10b</td>
</tr>
<tr>
<td><em>L. johnsonii</em> OCS41</td>
<td>Korean infant feces</td>
<td>28.15 ± 2.65bc</td>
<td>6.77 ± 1.36cd</td>
</tr>
<tr>
<td><em>L. gasseri</em> K43</td>
<td>Korean infant feces</td>
<td>16.85 ± 3.95d</td>
<td>7.38 ± 0.73bcd</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. **a,b,c,d** Mean values with different superscripts are significantly different as determined by Tukey’s test (*p < 0.05*).
and inhibition of reactive oxygen species (ROS) production resulting from enzyme-related oxidative stress. Because epigallocatechin gallate (EGCG), derived from green tea, attenuated the cytotoxicity induced by a lethal dose of ethanol and appeared to inhibit gamma-glutamyltransferase (GGT) activity,25) we evaluated the possibility that the total content of catechin GTE induced in fermentation with L. fermentum increased the viability of alcohol-treated HepG2 cells. Unexpectedly, there was no significant difference in catechin content between GTE and FGTE (316.19 ± 16.54 and 274.36 ± 24.44 mg catechin/g of green tea respectively). Fermentation processing by lactic acid bacteria is capable of promoting the degradation or modification of catechins in GTE. Hence we consider that modified GTE components (e.g., catechin) through L. fermentum fermentation may be critical to the hepatoprotective effect. Our ongoing study evaluates structural modifications of catechin fermented by L. fermentum OCS19 by 1H-nuclear magnetic resonance (NMR) methods.

### Hepatoprotective Effects of Probiotics, Green Tea, and a Fermented Product of it in Vivo

The blood alcohol concentration after oral administration of ethanol to water-fed mice reached a peak level of 0.4011% at 1 h and declined gradually thereafter (Table 3), but this group showed no significant differences in blood alcohol concentration at 1 and 3 h as compared with the sample-pretreated groups. At 6 h, however, the blood alcohol concentrations in the FGTE- and H. dulcis pretreated groups were significantly decreased relative to the water pretreated group (Table 3). Similarly, Choi et al., have reported that H. dulcis decreased the blood alcohol concentration by about 20% at 3 h. In the present study, FGTE decreased the blood alcohol concentration at 6 h to the same extent as H. dulcis. GTE also reduced the blood alcohol concentration, but to a lesser extent. Hence, elimination of alcohol in the FGTE-pretreated mice was more efficient than in the GTE-pretreated mice, suggesting that GTE associated activity is clearly enhanced by fermentation with L. fermentum.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood alcohol concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Water</td>
<td>0.4011 ± 0.0083NS</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>0.3916 ± 0.0164NS</td>
</tr>
<tr>
<td>GTE</td>
<td>0.3894 ± 0.0710NS</td>
</tr>
<tr>
<td>FGTE</td>
<td>0.3609 ± 0.0121NS</td>
</tr>
<tr>
<td>H. dulcis</td>
<td>0.3459 ± 0.0052NS</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Mean values with different superscripts are significantly different as determined by Tukey’s test (p < 0.05).

**Table 3.** Blood Alcohol Concentrations in Mouse Serum after Administration of Ethanol

![Fig. 2.](image)

**Fig. 2.** Effects of L. fermentum, GTE, FGTE, and H. dulcis on Biochemical Factors in Vivo.

ALT activity (A), AST activity (B), triglyceride levels (C), and total cholesterol levels (D) are shown for various groups of experimental mice. Each group of mice was given 3 g/kg ethanol after pretreatment with water, L. fermentum (10⁷ CFU/kg), GTE (50 mg/kg), FGTE (50 mg, green tea/kg + 10⁸ CFU/kg) or H. dulcis (41 mg/kg). The control group received an isocaloric maltose solution (5.5 g/kg) instead of ethanol. *abcd* Mean values with different superscripts are significantly different by Tukey’s test (p < 0.05).
induced hyperlipidemia. Levels of total cholesterol, however, were similar among all groups (Fig. 2D).

The increased levels of serum triglycerides and cholesterol resulting from ethanol absorption might have been due to any of several processes, including increased availability of free fatty acids and l-glycerophosphate, increased secretion of very low density lipoprotein into the serum, and decreased removal of triglycerides and cholesterol from the serum due to diminished lipoprotein lipase activity. A reversal of any of these processes might have been responsible for the observed ability of *L. fermentum*, GTE, and *H. dulcis* pretreatment to decrease triglyceride levels in the mice following ethanol challenge.

**Histological analysis**

Ethanol can induce severe liver damage. Figure 3 shows clear-cut histopathological changes in the H&E-stained murine liver after acute ethanol exposure. Normal liver histology is shown in Fig. 3A. Swelling and hytropic degeneration of the hepatocytes around the central veins was observed in the water pretreated group following ethanol exposure (Fig. 3B) and, to a lesser degree, in the GTE pretreated group (Fig. 3D), but the hepatocytes in the *L. fermentum* pretreated group, and especially in the FGTE and *H. dulcis* pretreated groups, showed a morphology similar to that of the hepatocytes in the normal group (Fig. 3C, E, and F). Thus acute ethanol exposure caused degenerative morphological changes in the liver that were prevented to different degrees by pretreatment with *L. fermentum*, GTE, FGTE, and *H. dulcis*. The results of this experiment indicate that FGTE and *H. dulcis* protected the liver by limiting hepatocyte damage.

**Metabolizing hepatic alcohol enzyme activities**

ADH is an important liver enzyme that oxidizes alcohol, reducing its hepatic concentration. An increase in ADH activity is thus followed by a decrease in the ethanol concentration. ADH activity was significantly elevated in the livers of the water pretreated mice (304.1 ± 11.8 U/mg) as compared with the control group (220.7 ± 13.7 U/mg). The *H. dulcis* treated group showed the highest ADH activity (387.6 ± 11.3 U/mg). The FGTE group exhibited a similar elevation in enzyme activity (368.7 ± 10.7 U/mg) (Fig. 4A). ALDH activity did not differ appreciably among the groups (Fig. 4B).

A alcohol-induced cell damage is closely related to a reduction in alcohol metabolism. In accord with this, the enzyme activity of ADH decreased with increasing ethanol doses, and duration of exposure. High ADH and ALDH activity is in turn indicative of effective promotion of alcohol clearance from the blood serum. Because FGTE was almost as efficacious as *H. dulcis* in increasing ADH activity, administration of FGTE might rapidly ameliorate ethanol-induced hepatic damage by increasing its activity, enhancing alcohol metabolism and preventing the accumulation of acetaldehyde.

Among the multiple isozymes of ADH and ALDH in the liver, ADH1 and ALDH2 are present at high levels in terms of mRNA and protein expression. Hence the mRNA expression levels for these isoenzymes were explored in the livers of the experimental mice. A 1 h, exposure to ethanol induced expression of the ADH1 and ALDH2 mRNAs in the water pretreated versus the control murine liver, by 17% and 15% respectively (Fig. 5). On the other hand, the mRNA level of ADH1 was elevated to 1.48-fold of the value for the control group by FGTE pretreatment (Fig. 5A). However, the mRNA expression of ALDH2 did not show any significant difference among groups (Fig. 5B). Therefore, increased ADH1 expression, as well as enhanced ADH1 activity, probably accounted for the decreased blood alcohol concentration observed in the FGTE pretreated group versus the water, *L. fermentum* and GTE pretreated groups (Table 3).
In conclusion, our results indicate that FGTE has a protective effect against alcohol-induced liver damage, as evidenced by its ability to lower the blood alcohol concentration after ethanol challenge, as well as its ability to decrease the levels of ALT and triglycerides. FGTE also elevated ADH activity and mRNA expression in the liver. In sum, FGTE can play a beneficial role in the treatment of alcohol-induced liver damage.

Acknowledgments

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References