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Effect of *Lactobacillus fermentum* MG590 on Alcohol Metabolism and Liver Function in Rats

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Abstract Alcohol consumption has numerous health consequences for the human body. For example, heavy drinking on a daily basis causes liver diseases, and certain products such as acetaldehyde produced from alcohol metabolism are more toxic than alcohol itself. Accordingly, the current study evaluated the role of *Lactobacillus fermentum* MG590 to enhance the removal of the toxic effect of alcohol in alcohol metabolism. The maximum activities of the alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) by *L. fermentum* MG590 were observed after 6 h of culture. The production of ADH and ALDH by *L. fermentum* MG590 was also confirmed by SDS-PAGE. Six hours after the addition of alcohol to a culture broth of *L. fermentum* MG590, the alcohol concentration decreased from 7.5 to 2.7%. From an *in vitro* evaluation based on hepatocytes, the viability of hepatocytes in a medium containing alcohol and the cytosol of *L. fermentum* MG590 was higher than that in a medium containing only alcohol. From an *in vivo* test using SD rats fed a 22% alcoholic drink, the blood alcohol concentration (BAC), glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) in the rats fed a medium containing *L. fermentum* MG590 were lower than those in the rats fed a medium containing only the alcohol drink. These results demonstrate that the ADH and ALDH produced by *L. fermentum* MG590 play an important role in detoxicating alcohol *in vivo*. Therefore, a fermentation broth of *L. fermentum* MG590 could be used as an effective alcohol detoxification drink.

Key words: *L. fermentum* MG590, ADH, ALDH, liver, BAC, GOT, GPT

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After drinking alcoholic beverages, the alcohol is absorbed into the blood and diluted by the total body water. The bulk of the ingested alcohol (95–98%) is oxidized into acetaldehyde and acetate, while the rest (2–5%) is excreted through breath, urine, and sweat. The oxidation of alcohol occurs primarily in the liver. The major pathway for alcohol metabolism involves the production of alcohol dehydrogenase (ADH), which converts alcohol into acetaldehyde through an enzymatic oxidation process. Acetaldehyde is highly toxic to the body even at a low concentration. However, aldehyde dehydrogenase (ALDH) rapidly oxidizes acetaldehyde into acetate [18, 39], which is then oxidized into carbon dioxide and water, principally in the extrahepatic muscle tissues [9].

Regular alcohol consumption can have numerous consequences, both beneficial and detrimental, on the health of the drinker. For example, alcohol consumption can protect the body against certain types of heart disease and stroke, whereas heavy drinking has been associated with liver diseases. Furthermore, some products generated during alcohol metabolism are more toxic than alcohol itself. The liver is particularly susceptible to alcohol-related injuries, as it is the organ that metabolizes alcohol [3, 18, 39].

Numerous lactic acid bacteria are currently used in the prevention and treatment of diseases, and the important species include *Lactobacillus acidophilus*, *L. bifidus*, *L. bulgaricus*, *L. casei*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*. *L. salivarius*, *Streptococcus thermophilus*, and *Streptococcus faecium*. These lactic acid bacteria inhibit the proliferation and activities of putrefactive and pathogenic bacteria in several ways. In particular, *L. fermentum* protects the liver from alcohol and enhances specific liver functions [26]. Accordingly, the current study was carried out to evaluate the role of *L. fermentum* MG590 to decrease the toxic effect of alcohol in alcohol metabolism.

MATERIALS AND METHODS

Cell Culture

L. fermentum MG590 was cultured on an MRS broth medium (pH 6.5) at 37°C. The growth of *L. fermentum* MG590 was recorded by periodic sampling of the culture broth. The cell densities were measured using a spectrophotometer ($A_{660\ nm}$). The cells were harvested by centrifuging the culture broth, then suspended in 10 mM sodium phosphate buffer (pH 7.5). An aliquot of the bacterial suspension was sonicated in an ice bath for 3 min, and then centrifuged at 16,000 rpm and 4°C for 90 min to obtain the cytosol (cell-free supernatant), which was finally stored at 4°C until further analysis [19, 23].

Enzymatic Assays of ADH and ALDH

To measure the ADH and ALDH activities, the culture broth of *L. fermentum* MG590 after 4 h of culture was supplemented to make three different medium conditions: supplementation with 10% alcohol, 10% D.W., and no supplementation as the control. The ADH and ALDH activities were then measured 2 h after the addition of the supplements. The ADH and ALDH measurements were based on absorbance of NADH at 340 nm. One unit was defined as the amount of enzyme that converts 1 μmole of alcohol into acetaldehyde per minute at pH 8.8 and 25°C. For measuring the ALDH activity, the reduction of NAD^+ was detected at 340 nm after the addition of acetaldehyde. One unit was defined as the amount of enzyme needed to oxidize of 1.0 μmole of acetaldehyde into acetic acid per minute at pH 8.0 and 25°C [5, 22, 28, 42, 43]. The protein content of the enzyme source was determined by the Bradford assay method using bovine serum albumin as the standard. The ADH and ALDH activities were calculated as units/mg protein/min [22, 23].

SDS-PAGE Analysis

The ADH and ALDH produced by *L. fermentum* MG590 were analyzed by SDS-PAGE. The loading samples were obtained using the same methods as for the enzyme activity test. The SDS-PAGE was run in a gel containing 15% acrylamide and 0.1% SDS according to Laemmli [14]. Twenty μl of samples were added to a 5× sample buffer, and the mixture was denaturated at 100°C for 10 min [4]. The ADH and ALDH produced by Baker's yeast (Sigma Chemical, Missouri, U.S.A.) were used as the standards.

Gas Chromatography (GC)

The alcohol eliminated during the incubation of *L. fermentum* MG590 was immediately analyzed by gas chromatography using a GC-MS system (GC: GC-17A, MS: QP-5050A, Shimadzu). Alcohol was added to the culture broth after 4 h of culture, and samples were then taken at 2 h intervals over a 6 h period. The samples were centrifuged at 8,000 rpm and 4°C for 15 min to obtain the supernatant that was used for the GC assay. The chromatographic separation was performed by split mode injection (split ratio, 20:1) of 2 μl of the samples into the column (60 m×0.32 mm I.D and 1 mm film thickness, DB-1MS, J&W). The injector temperature was 180°C and initial oven temperature was 50°C. The temperature was programmed as follows: 5°C/min up to 100°C and 10°C/min up to 200°C [2, 11, 12, 20, 22, 23].

Isolation of Rat Hepatocytes and *In Vitro* Evaluation of Effect of *L. fermentum* MG590 Using Hepatocytes

Hepatocytes were harvested from male Sprague-Dawley rats (8-week old) using a two-step *in situ* collagenase perfusion technique, modified from the method described by Seglen [8, 13, 15, 30]. The liver was perfused by 250 ml of a perfusion buffer (NaCl 9 g/l, KCl 0.42 g/l, glucose 0.99 g/l, $NaHCO_3$ 2.1 g/l, HEPES 4.77 g/l) at a flow rate of 27 ml/min for 10 min. After the initial flushout, perfusion buffer supplemented with collagenase (0.5 g/l) was perfused at a flow rate of 25 ml/min for 10 min, and then the liver was filtered through a filter gauge and the hepatocytes were obtained by percoll gradient centrifugation. The viability of the isolated hepatocytes in the current study was over 85%, according to the trypan blue exclusion method. The hepatocytes were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with insulin (10 mg/l), epidermal growth factor (EGF, 1 μg/l), glucagon (7 μg/l), hydrocortisone (7.5 mg/l), L-proline (60 mg/l), sodium bicarbonate (4.5 g/l), penicillin (100 unit/l), streptomycin (100 mg/l), and 10% FBS (pH 7.4) in 6-well plates in a humidified atmosphere of 10% CO_2 and 90% air at 37°C. The hepatocytes were divided into four groups with different supplements in the DMEM: no supplement (control), 10% alcohol, 10% cytosol of *L. fermentum* MG590 plus 10% alcohol, and 20% cytosol of *L. fermentum* MG590 plus 10% alcohol. The cultured hepatocytes were observed daily using light microscopy over 7 days, and the spent media were completely replaced with fresh media everyday [11, 12].

Determination of Blood Alcohol Concentration (BAC) for Evaluating *In Vivo* Effect of *L. fermentum* MG590 in SD Rats

To determine the effect of the ADH and ALDH activities from *L. fermentum* MG590 on *in vivo* alcohol metabolism, SD rats (8-week old) were fed a culture broth of *L. fermentum* MG590 including 22% alcoholic drink. Based on the previous report by Dubowski *et al.* [7, 41], blood was then taken 2 h (120 min) after the oral administration. The rats were also fed various concentrations of *L. fermentum* MG590 to determine the optimum concentration of *L. fermentum* MG590. Two hours after the intake of

22% alcohol and the culture broth of *L. fermentum* MG590, trunk blood was collected from each rat at the time of sacrifice (with 5 min interval per rat). The blood samples were centrifuged at 3,000 rpm for 10 min to obtain the serum for measurement of BAC using an alcohol kit (Sigma Chemical, Missouri, U.S.A.) [10, 25].

GOT and GPT Activities in SD Rats fed *L. fermentum* MG590

Glutamic oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were determined by the methods of Reitman-Frankel using a kit (ASAN Co., Gyeonggi, Korea) and expressed as karmen units [27]. To measure the *in vivo* GOT and GPT activities, the SD rats were divided into three groups and fed different supplements: water was given to group 1, *L. fermentum* MG590 plus 22% alcohol was given to group 2, and 22% alcohol was given to group 3. GOT and GPT in the sera were obtained by the same method as used for the BAC assay. GOT and GPT activities were measured just before and 2 h after the oral administration of the supplements [4, 11, 12, 25, 33, 35].

RESULTS AND DISCUSSION

ADH and ALDH Activities in Culture of *L. fermentum* MG590

The maximum ADH and ALDH activity produced by *L. fermentum* MG590 was observed after 6 h of cultivation, as shown in Fig. 1. *L. fermentum* MG590 is a heterolactic fermentation type [19, 34] and the metabolites of the catabolic pathway are lactic acid, acetic acid, alcohol, and CO_2. The relative yields of acetic acid and alcohol are related to the availability of $NAD(P)^+$ in the cells [17, 29, 34]. Acetyl-CoA is converted into alcohol by ALDH and ADH in an alcohol-forming pathway. Indeed, the maximum enzyme activities of ADH (4.0 unit/mg protein of ADH)

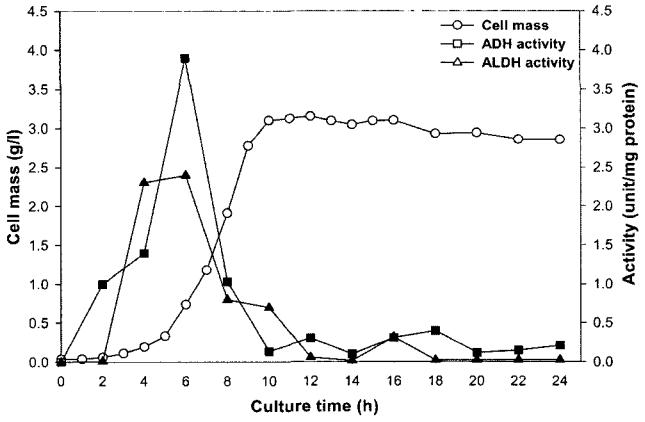

Fig. 1. Growth curve and ADH and ALDH activities of *L. fermentum* MG590.

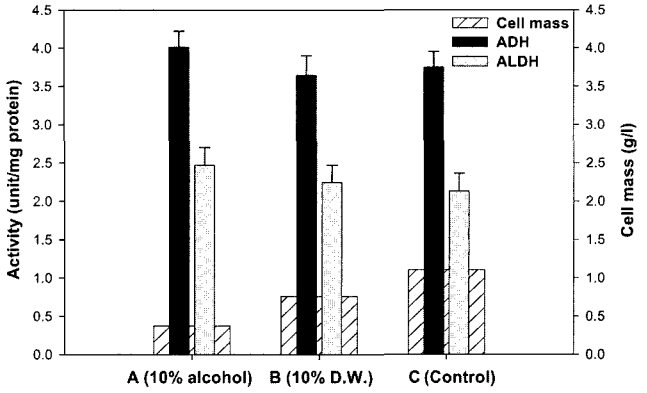

Fig. 2. ADH and ALDH activities produced by *L. fermentum* MG590.

Alcohol or D.W. was added to the culture broth after 4 h of culture. The ADH and ALDH activities were then measured 2 h after the addition of 10% alcohol (A) and D.W. (B) to the culture broth. The culture broth without any addition (C) was used as the control.

and ALDH (2.5 unit/mg protein of ALDH) in *L. fermentum* MG590 were exhibited in the early log phase (6 h). However, the enzyme activities decreased rapidly to 0.25 unit/mg protein of ADH and 0 unit/mg protein of ALDH in the stationary phase.

Therefore, 10% alcohol or 10% D.W. was added after 4 h of cultivation, because ADH and ALDH activities decreased after 6 h of cultivation (Fig. 1). As shown in Fig. 2, the ADH and ALDH activities in the culture broth containing 10% alcohol were higher than those in the control, although the cell mass in the culture broth was lower. As such, it is considered that the enzyme biosynthesis was increased to eliminate the alcohol added, instead of its utilization for cell growth.

SDS-PAGE Analysis

The ALDH and ADH produced from *L. fermentum* MG590 were analyzed by SDS-PAGE, as shown in Fig. 3. The molecular weight of the ADH from *L. fermentum* MG590 was about 40 kDa (the bands for lanes D and E), which is similar to that from *Zymomonas mobilis* [31], yet smaller than the standard ADH from Baker's yeast (the band for lane C). The ADH from yeast is a tetrameter of about 150 kDa that contains zinc and has an amino acid sequence very similar to the dimeric ADH from mammalians [36, 37]. Under the denaturing conditions of SDS-PAGE, the molecular mass of the ADH from yeast is about 43 kDa [6]. As such, the molecular weight of the ALDH from *L. fermentum* MG590 was found to be about the same size as the standard ALDH (Baker's yeast), about 55 kDa and 66 kDa (the bands for lanes D and E) (Fig. 3). Several researchers have already demonstrated that *S. cerevisiae* produces five different ALDHs with a total molecular mass of 200 kDa and average molecular mass for the subunits of the cytoplasmic ALDH of 54 kDa [21, 38, 40],

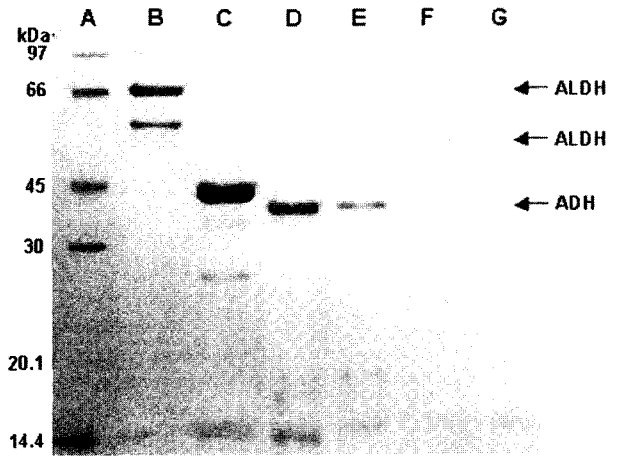

Fig. 3. SDS-PAGE of ADH and ALDH in *L. fermentum* MG590 cytosol.
A: Marker (low molecular weight); B: standard ALDH (Baker's yeast); C: standard ADH (Baker's yeast); D: addition of 10% ethanol to culture broth; E (control): culture broth (0% ethanol); F: *L. acidophilus* (minimal enzyme activity); G: negative control with *L. gasseri* (no enzyme activity).

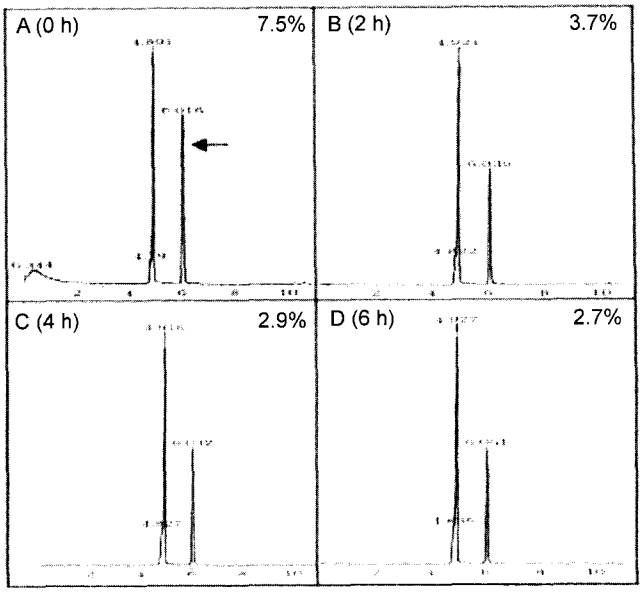

Fig. 4. Gas chromatogram of alcohol in *L. fermentum* MG590.
Alcohol was added to the culture broth after 4 h of cultivation. Samples were then taken and analyzed at 0 h (A), 2 h (B), 4 h (C), and 6 h (D) after the addition of 10% alcohol.

while the ALDH from other bacteria has been found to be 55 kDa [24, 32]. Furthermore, the major alcohol metabolizing enzymes, such as ADH and ALDH, also include a genetic polymorphism that alters the rate of oxidation of toxic metabolites such as acetaldehydes, and are expressed with various molecular weights [1]. The molecular weights of the ADH and ALDH from *L. fermentum* MG590 were similar to those of the ADH and ALDH from standard yeast. However, the molecular weight of *L. fermentum* MG590 was found to be slightly smaller than that of yeast. The ADH band for lane D (addition of alcohol) was thicker than that for lane E (no addition of alcohol), as shown in Fig. 3, indicating that the supplementation of alcohol induced the production of ADH in *L. fermentum* MG590. Lane F with the cytosol of *L. acidophilus* (slight enzyme activity) showed faint bands of ADH and ALDH. Lane G as the negative control with the cytosol of *L. gasseri* (no enzyme activity) revealed no bands, indicating no production of ADH and ALDH. Accordingly, these results suggest that *L. fermentum* MG590 produced ADH and ALDH to eliminate the alcohol, and the biosynthesis of ADH and ALDH was inducible by the addition of alcohol.

Gas Chromatography

As shown in Fig. 4, the amounts of alcohol in the culture medium with and without *L. fermentum* MG590 were

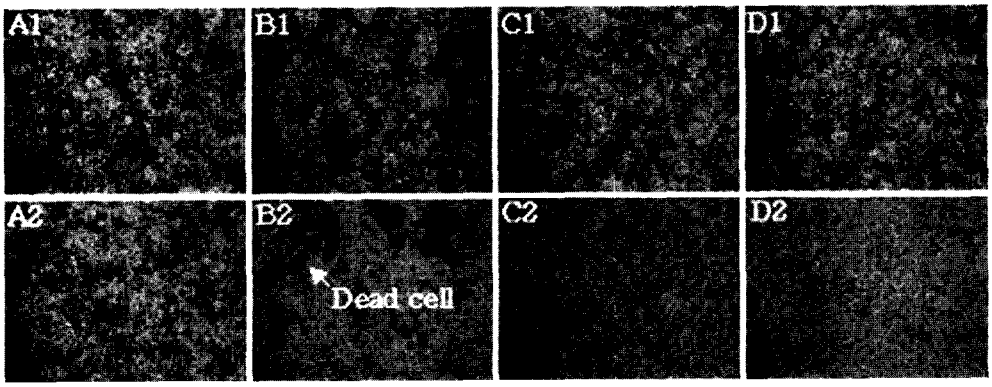

Fig. 5. *In vitro* test of *L. fermentum* MG590 using hepatocytes.
The hepatocytes were cultured in DMEM supplemented with different components: no addition as the control (A), 10% alcohol (B), 10% cytosol from *L. fermentum* MG590 plus 10% alcohol (C), and 20% cytosol from *L. fermentum* MG590 plus 10% alcohol (D). The cultured hepatocytes were observed daily using light microscopy over 7 days. 1: 0 day after addition; 2: 7 days after addition.

analyzed by gas chromatography [11, 12]. Alcohol was added to the culture broth after 4 h of cultivation. Six h after the addition of alcohol, the concentration decreased from 7.5 to 2.7% in the culture medium with *L. fermentum* MG590. The maximum reduction period of alcohol was from 0 h to 2 h after the addition of alcohol, which also corresponded to the period of maximum enzyme activity after 4 h to 6 h of cultivation, as shown in Fig. 1. Meanwhile, in the culture medium without *L. fermentum* MG590, the alcohol concentration remained changed. As such, the rapid decrease in the alcohol concentration was confirmed to be correlated to the ADH and ALDH activity.

In Vitro Evaluation of Effect of *L. fermentum* MG590 on Hepatocytes

The hepatocytes cultured in 6-well plates containing alcohol and the cytosol of *L. fermentum* MG590 (Figs. 5 C2 and 5 D2) exhibited healthier adhesion to the plate than those in the medium containing only alcohol (Fig. 5 B2) after 7 days of culture. Most of the hepatocytes (Fig. 5 B2) were detached from the plate, and the dead cells were determined by the trypan blue test. The hepatocytes were injured by the addition of alcohol, whereas viable cells were maintained by the addition of *L. fermentum* MG590, as shown in Figs. 5 C2 and 5 D2, similar to the control (Fig. 5 A2). Viable hepatocytes were also observed in the media containing 10% (Fig. 5 C2) and 20% (Fig. 5 D2) cytosol from *L. fermentum* MG590 with 10% alcohol. Hence, increasing the concentration of *L. fermentum* MG590 had no significant effect on the hepatocytes. However, the culture in the medium with only alcohol exhibited high mortality, indicating that *L. fermentum* MG590 was effective in reducing liver injury and maintaining the viability of hepatocytes.

Determination of BAC for *In Vivo* Evaluation of Effect of *L. fermentum* MG590 in SD Rats

To evaluate the *in vivo* effect of *L. fermentum* MG590 on alcohol metabolism, SD rats were fed a culture broth containing *L. fermentum* MG590 (0.75 g/l) and 22% alcoholic drink. Two h after the intake, the BAC of the rats fed the 22% alcoholic drink with and without *L. fermentum* MG590 was 0.005% and 0.070%, respectively (Fig. 6A).

The rats were fed various concentrations of *L. fermentum* MG590 to determine the optimum concentration. However, increasing the concentration of *L. fermentum* MG590 from 0.4 g/l to 10.0 g/l had no significant effect on decreasing the BAC, as shown in Fig. 6B. Therefore, a minimum amount of *L. fermentum* MG590 broth (0.4 g/l) was found to be sufficient to reduce the BAC in the *in vivo* test.

GOT and GPT in SD Rats as *In Vivo* Evaluation of Addition of *L. fermentum* MG590

GOT and GPT are both produced by the liver and are required to metabolize amino acids. However, a large amount of transaminase (GOT and GPT) is released into the serum, when liver cells are damaged [16]. Therefore, liver cell damage can be determined by measuring the levels of GOT and GPT.

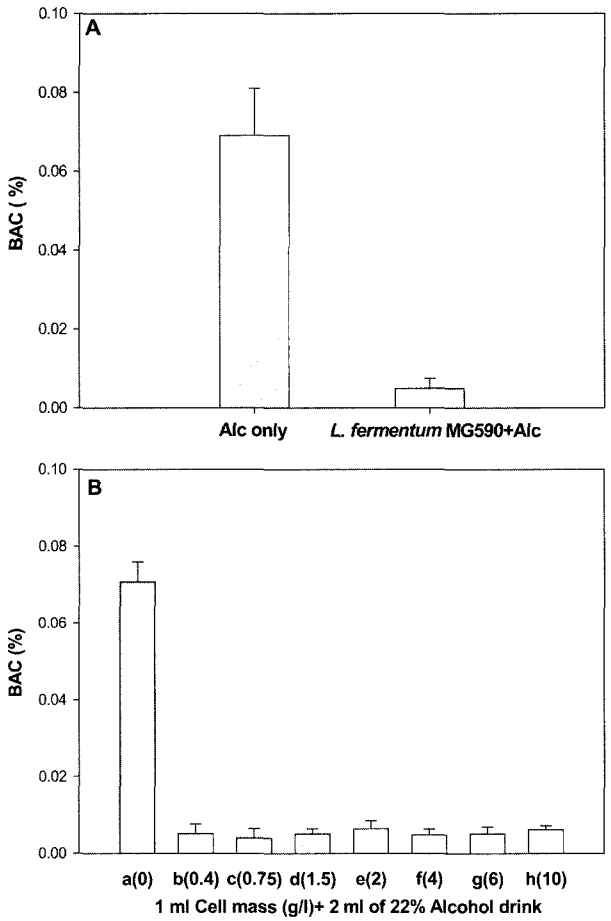

Fig. 6. Measurement of BAC in SD rats fed *L. fermentum* MG590 and/or 22% alcohol drink.
A: Alc only, 1 ml unused medium+2 ml 22% alcohol drink; *L. fermentum* MG590+Alc, 1 ml *L. fermentum* MG590+2 ml 22% alcohol drink. B: a (0): 1 ml unused medium+2 ml 22% alcohol drink (control), b (0.4): 1 ml *L. fermentum* MG590 (0.4 g/l)+2 ml 22% alcohol drink; c (0.75): 1 ml *L. fermentum* MG590 (0.75 g/l)+2 ml 22% alcohol drink; d (1.5): 1 ml *L. fermentum* MG590 (1.5 g/l)+2 ml 22% alcohol drink; e (2): 1 ml *L. fermentum* MG590 (2.0 g/l)+2 ml 22% alcohol drink; f (4): 1 ml *L. fermentum* MG590 (4 g/l)+2 ml 22% alcohol drink; g (6): 1 ml *L. fermentum* MG590 (6 g/l)+2 ml 22% alcohol drink; h (10): 1 ml *L. fermentum* MG590 (10 g/l)+2 ml 22% alcohol drink.

The karmen units of GOT and GPT measured before feeding the supplement to the rats were 24.4 and 32.0 for group 1 (water), 21.6 and 27.7 for group 2 (*L. fermentum* MG590+Alc), and 20.5 and 31.3 for group 3 (Alc only), respectively, as shown in Fig. 7A. Then 3 ml of water was given to group 1, 1 ml of *L. fermentum* MG590 and 2 ml of 22% alcoholic drink to group 2, and 1 ml of the medium without *L. fermentum* MG590 and 2 ml of 22% alcoholic drink to group 3. As shown in Fig. 7B, 2 h after feeding,

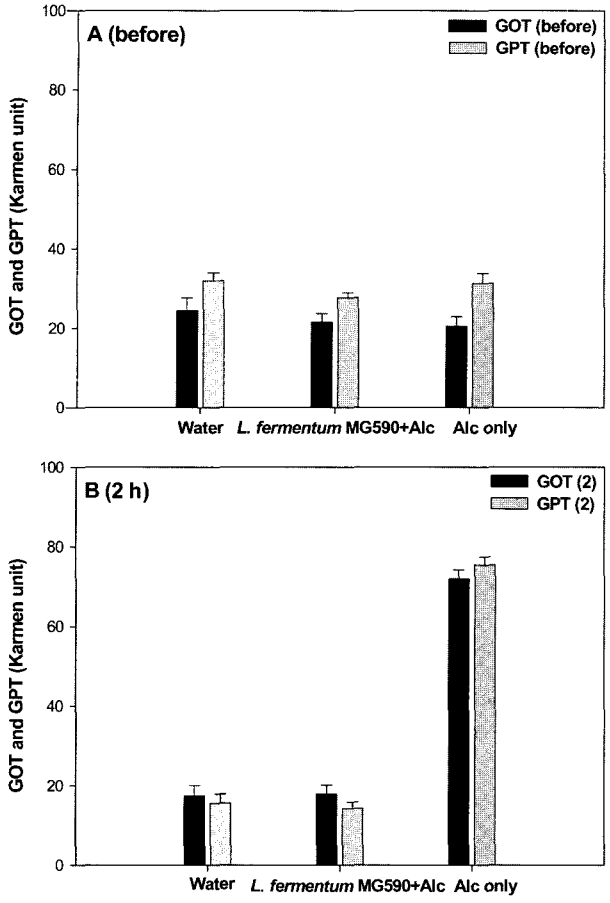

Fig. 7. *In vivo* test of GOT and GPT activities in SD rats fed *L. fermentum* MG590 and/or 22% alcohol drink.
The SD rats were fed different supplements: feeding 3 ml water (Control), 1 ml *L. fermentum* MG590 containing 2 ml of 22% alcohol (*L. fermentum* MG590+Alc), and 3 ml of 22% alcohol only (Alc only). The GOT and GPT values were determined before (A) and 2 h after (B) the oral administration of the supplements.

the GOT and GPT values in group 3 dramatically increased, while in groups 1 and 2, the GOT and GPT values decreased. The GOT and GPT values for group 2 fed on *L. fermentum* MG590 with alcohol were similar to those for group 1 as the control. In fact, the GOT and GPT values for group 2 decreased from 21.6 and 27.7 to 17.9 and 14.3 karmen units, respectively, even with the feeding of alcohol, indicating that *L. fermentum* MG590 enhanced both the liver function and the metabolism of alcohol.

In summary, the ADH and ALDH from *L. fermentum* MG590 were found to have a considerable effect on alcohol metabolism, thereby reducing the blood alcohol concentration. In addition, *L. fermentum* MG590 also protected the liver and maintained the liver functions against alcohol. Therefore, to the best of our knowledge, this is the first report on the effects of LABs or enzymes produced from LABs on alcohol metabolism and the real effects of the ADH and ALDH from the LAB, *L. fermentum* MG590, on reducing alcohol *in vivo*.

Acknowledgments

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