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Characteristics and *in vitro* Anti-diabetic Properties of the Korean Rice Wine, Makgeolli Fermented with *Laminaria japonica*

Jae-Suk Choi[1,2], Hyo Ju Seo[1], Yu-Ri Lee[2], Su-Jung Kwon[2], Sun Hwa Moon[1], Sun-Mee Park[3], and Jae Hak Sohn[1,2]

[1]RIS Center, [2]Department of Bio-Food Materials, and [3]HACCP Training Center, Silla University, Busan 617-736, Korea

ABSTRACT: New *in vitro* anti-diabetes makgeolli was produced from rice by adding various quantities of *Laminaria japonica*, and the fermentation characteristics of the *L. japonica* makgeolli during the fermentation process were investigated. The contents of alcohol and reducing sugar, and viable count of yeast, of *L. japonica* makgeolli were not significantly changed when the proportion of *L. japonica* was increased. The total acid content decreased with an increase in *L. japonica* concentration; the pH and total bacterial cell count increased in proportion with the increase in *L. japonica* concentration. The *L. japonica* makgeolli contents of free sugars, such as fructose, glucose, and sucrose, and of organic acids, such as acetic acid, citric acid, succinic acid, and lactic acid, were altered during fermentation and showed various patterns. The effects of the quantity of *L. japonica* added on the acceptability and anti-diabetes activities of *L. japonica* makgeolli were also investigated. In a sensory evaluation, *L. japonica* makgeolli brewed by adding 2.5 or 5% *L. japonica* to the mash showed the best overall acceptability; the 12.5% *L. japonica* sample was least favored due to its seaweed flavor. *L. japonica* addition did not increase the peroxynitrite-scavenging activity of makgeolli. *L. japonica* makgeolli showed potent anti-diabetes activity, particularly that containing >7.5% *L. japonica*. Therefore, *L. japonica* makgeolli may represent a new functional makgeolli with anti-diabetes properties.

Keywords: makgeolli, rice wine, *Laminaria japonica*, quality characteristics, anti-diabetes activity

INTRODUCTION

Makgeolli (also known as takju) is the oldest Korean traditional rice wine. It contains 6~7% alcohol and has long been brewed classically using nuruk or koji, cooked rice and flour, and yeasts. Unlike other alcoholic beverages, makgeolli is highly nutritious and functional because it contains proteins, sugars, vitamins, bioactive compounds, and various organic acids (1,2). It was recently reported that makgeolli also has various physiological effects; e.g., antioxidant (3), anti-hypertensive (4), anti-diabetes (5), and anti-cancer (6) activities.

Several research groups have sought to identify methods by which the quality of makgeolli can be improved, and have investigated the changes in the microbe content and enzyme activity during fermentation, the nutrient content and acceptability characteristics, utilization of raw materials, standardization of the manufacturing process, storage, and marketing (1,2,7).

The size of the market for makgeolli has increased gradually, reaching ~270 million US dollars in 2012 (8). However, several problems have occurred, such as a lack of unique characteristics, and inferior acceptability and functionality. Therefore, development of new Korean rice wines with excellent acceptability and functionality is necessary.

In previous studies, various types of makgeolli were developed using ginseng (9), pears (10), blueberries (11), chestnut peel (12), cucumbers (13), and banana (14). However, while their quality characteristics during fermentation were determined, their functionalities were not assessed.

L. japonica is commonly used as a foodstuff in Korea, Japan, and China and has been reported to contain various nutrients; such as alginate, proteins, free amino acids, sugars, and minerals (15). In addition, *L. japonica* has been reported to exhibit various properties beneficial to health, such as antimicrobial (16), antioxidant (17), and anti-inflammatory (18) activities. In addition, *L. japonica* has antioxidant (19,20), ant-diabetic (21), anti-cancer (22), hypolipidemic (23), and anti-hypertensive (24) effects.

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Correspondence to Jae Hak Sohn, Tel: +82-51-999-5629, E-mail: jhsohn@silla.ac.kr

L. japonica is thus a promising ingredient with various biological activities, use of which could increase the physiological functionality of Korean traditional rice wine. In this paper, we describe the optimal fermentation conditions for the development of a new functional *L. japonica* makgeolli. In addition, we describe the sensory qualities and the antioxidant, anti-hypertensive and anti-diabetes activities of *L. japonica* makgeolli brewed using 2.5∼12.5% *L. japonica* in the mash.

MATERIALS AND METHODS

Materials

Rice (*Oryza sativa* L.) was purchased from Hamyang Agricultural Cooperative Association (Hamyang, Korea). The brown seaweed *L. japonica* from which holdfasts had been removed was cultivated for 80∼90 days at a seaweed aquaculture farm owned by Gijang Fishery Association Corporation located in Sirang, Gijang on the east coast of Busan, Korea. The seaweed tissues were washed with tap water to remove salt, epiphytes, and sand; dried for 1 day at room temperature; and then ground to a powder (200 mesh) in a milling machine (HJM-15100, Hansung Pulverizing Machinery Co. Ltd., Gwangju, Korea). Koji (*Aspergillus kawachii*), purchased from Chungmu Balhyo Co. Ltd. (Ulsan, Korea) and cultivated for 2 days in steamed flour) was provided by Dongbaek Yangjo Co. Ltd. (Busan, Korea). For mother brew (Mitsul), the yeast *Saccharomyces cerevisiae* (strain no. RIS-fj-38), selected and maintained at the RIS Center, Silla University (Busan, Korea), was used.

Preparation of mother brew

Mother brew was prepared as follows: koji (100 g), sterilized water (200 mL) and *S. cerevisiae* (5 mL) that had been cultured in potato dextrose broth (PDB; Difco, Sparks, MD, USA) at 25°C for 2 days were mixed and cultured at 25°C for 2 days.

Preparation of mash and *L. japonica* makgeolli fermentation

The fermentation procedures were divided into two steps: first, yeast proliferation; and second, saccharification and alcohol fermentation. In the first step, koji (900 g), sterilized water (2.4 L), and cultured mother brew (300 mL) were mixed and fermented at 25°C for 2 days. To prepare mash, washed non-glutinous rice (4.8 kg) was soaked for 2.5 h, drained for 30 min, steamed for 45 min. After rapid cooling, the steamed rice and 7.2 L of water were added to the product of the first step, and were then were mixed and fermented in a 20-L sterile glass container at 25°C for 7 days in the second step. To produce *L. japonica* makgeolli, 0, 2.5, 5, 7.5, 10, and 12.5% (w/w) *L. japonica* powder was added based on the starchiness during the second step of fermentation. After 7 days, the fermentation broth was strained through a sieve to produce traditional, turbid rice wine.

Preparation of *L. japonica* makgeolli extract

To investigate the antioxidant, anti-diabetes, and anti-hypertensive activities of *L. japonica* makgeolli, an *L. japonica* makgeolli extract was prepared. First, *L. japonica* makgeolli (7,864±926 mL) was fractionated into supernatant (7,034±1,104 mL) and precipitated (828±298 mL) by centrifugation at 4,500 *g* for 10 min. One liter of supernatant was evaporated at reduced pressure to ∼10% of the initial volume, yielding a brownish gummy residue that was freeze-dried for 3 days to a moisture content of ∼0.8%. The freeze-dried supernatant was ground in a mill and passed through a 200-mesh sieve. The sieved material (104±9.2 g) was stored at −20°C until use.

General analysis

The pH and alcohol, total acid, reducing sugar, organic acid, and free sugar contents of the samples were monitored by periodic sampling at 24-h intervals throughout the fermentation process. Alcohol concentration was measured using a 7890A Gas Chromatography System (Agilent Technologies, Santa Clara, CA, USA) with a headspace sampler, a flame ionization detector (FID), and an HP-FFAP gas chromatography column (30 m; i.d. 0.32 mm; film thickness 0.50 μm). Ultra-pure nitrogen was used as the carrier gas at a flow rate of 16.41 mL/min. The injector temperature was 230°C. The column conditions were 10 min at 60°C with the FID at 230°C. pH was measured using a pH meter (Fisher Scientific, Pittsburgh, PA, USA) and the total acid content was estimated (as titratable acidity) after titration to pH 7.0 with 0.1 N NaOH. Acetic acid content (%) was then calculated based on the total acid content. The reducing sugar content was determined by the 3,5-dinitrosalicylic acid (DNS) method (25) using glucose as the standard. Detection was performed at 575 nm using an ultraspectrophotometer (Ultrospec 3300 pro, Biochrom Ltd., Cambridge, England).

Free sugar and organic acid determination

To quantify free sugars (glucose, fructose, and sucrose), a high-performance liquid chromatography (HPLC) system equipped with a μ-Bondapak/carbohydrate analysis column was used as described by Kennedy et al. (26). Free sugar content was expressed as % (g/100 g). To determine the organic acid content of the makgeolli, 5 mL of each makgeolli supernatant were mixed with 45 mL of 0.1% formic acid and analyzed in terms of organic acid (acetic acid, citric acid, succinic acid, and lactic acid) contents using an HPLC system (Agilent 1200 series,

Agilent Technologies) with a Gracesmart™ column (4.6×250, 5 mm) by the method of Romero Rodriguez et al. (27).

Color determination

The color of the *L. japonica* makgeolli was assessed using a color difference meter (CM-700d Spectrophotometer; Konica Minolta Sensing, Inc., Tokyo, Japan). Color was expressed using Hunter system values: *L* (lightness), *a* (redness), and *b* (yellowness). The Hunter values were monitored using the SpectraMagic software (version 2.11; Minolta CyberChrome, Inc., Osaka, Japan). The overall color difference (ΔE) was calculated using the equation $\Delta E=[(\Delta L)^2+(\Delta a)^2+(\Delta b)^2]^{1/2}$.

Total bacterial count and total yeast count

For enumeration of microorganisms, after serial 10-fold dilutions of makgeolli with saline, 1 mL of each dilution was spread on 3M Petrifilm™ Bacterial Count Plates (3M, St. Paul, MN, USA). The agar plates were then incubated at 37°C under aerobic conditions for 48 h. Yeast colonies were cultured on potato-dextrose agar at 25°C under aerobic conditions for 5~7 days. Total bacteria and yeast viable counts were calculated as colony-forming units (CFU) per gram in independent triplicate experiments.

Sensory evaluation

Sensory evaluation of the *L. japonica* makgeolli was conducted by 12 trained sensory panels according to the quantitative descriptive analysis (QDA) method (28,29). The panels evaluated the taste, odor, and acceptability of the makgeolli on a scale of 1 to 9, where 9 is the best score. The means were obtained and plotted as a polygonal graph. Overall acceptability according to taste and odor was evaluated by scoring from 1 (extremely dislike) to 9 (extremely like). Refreshment was defined as the sensory properties of carbonating, energizing, and thirst quenching, which are intimately related to mouth wetting.

Peroxynitrite-scavenging activity

An Evans Blue bleaching assay was used to measure peroxynitrite ($ONOO^-$)-scavenging activity (30). The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, various concentrations of makgeolli extract, and 1 mM peroxynitrite in a final volume of 1 mL. After incubation for 30 min, the absorbance was measured at 611 nm. The percentage scavenging of $ONOO^-$ was calculated by comparison of the test and blank samples. The effect was compared with the standard antioxidant penicillamine.

Inhibition of angiotensin-converting enzyme (ACE)

ACE activity was assayed by the method of Cushman and Cheung (31). Captopril (C4042, Sigma, St. Louis, MO, USA) was used as the positive control.

Anti-diabetes activity

Protein tyrosine phosphatase 1B (PTP1B; human, recombinant) was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Enzyme activity was measured in a reaction mixture containing 2 mM *p*-nitrophenyl phosphate (*p*NPP) in 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT). The reaction mixture was placed in a 30°C incubator for 30 min, and the reaction was terminated by the addition of 1 N NaOH. The amount of *p*-nitrophenol produced was estimated by measuring the increase in absorbance at 405 nm. The non-enzymatic hydrolysis of 2 mM *p*NPP was corrected by measuring the increase in absorbance at 405 nm in the absence of PTP1B enzyme (32,33).

Calculation of IC_{50} by linear interpolation

IC_{50} was calculated using linear interpolation. The equation is as follows:

IC_{50}=[(50%-low percentage)/(high percentage-low percentage)]×(high concentration-low concentration)+ low concentration

Statistical analyses

Analysis of variance (ANOVA) was used to determine significant differences ($P\leq0.05$) among makgeolli with different *L. japonica* concentrations (2.5, 5, 7.5, 10, and 12.5% (w/w)) by each analytical characteristic and makgeolli with no *L. japonica*. ANOVAs of bacterial count, yeast count, and Hunter's color value *L*, *a*, and *b* attributes of each makgeolli with different *L. japonica* concentrations were also performed. The experiments were performed in triplicate, and microbial counts were performed in duplicate (n=6). Sensory tests including flavor, seaweed flavor, sweetness, bitterness, sourness, and overall acceptability were carried out once, and attributes were evaluated twelve times (n=12). Means and standard deviations for analytical characteristics, microbiological analysis, and sensory evaluation were calculated. The differences among samples were determined with Duncan's multiple-range test and considered statistically significant at the level of $P<0.05$. All statistical analyses were conducted with SPSS ver. 10 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Alcohol fermentation characteristics

The effects of the quantity of *L. japonica* added on the alcohol fermentation of makgeolli were investigated (Fig. 1A).

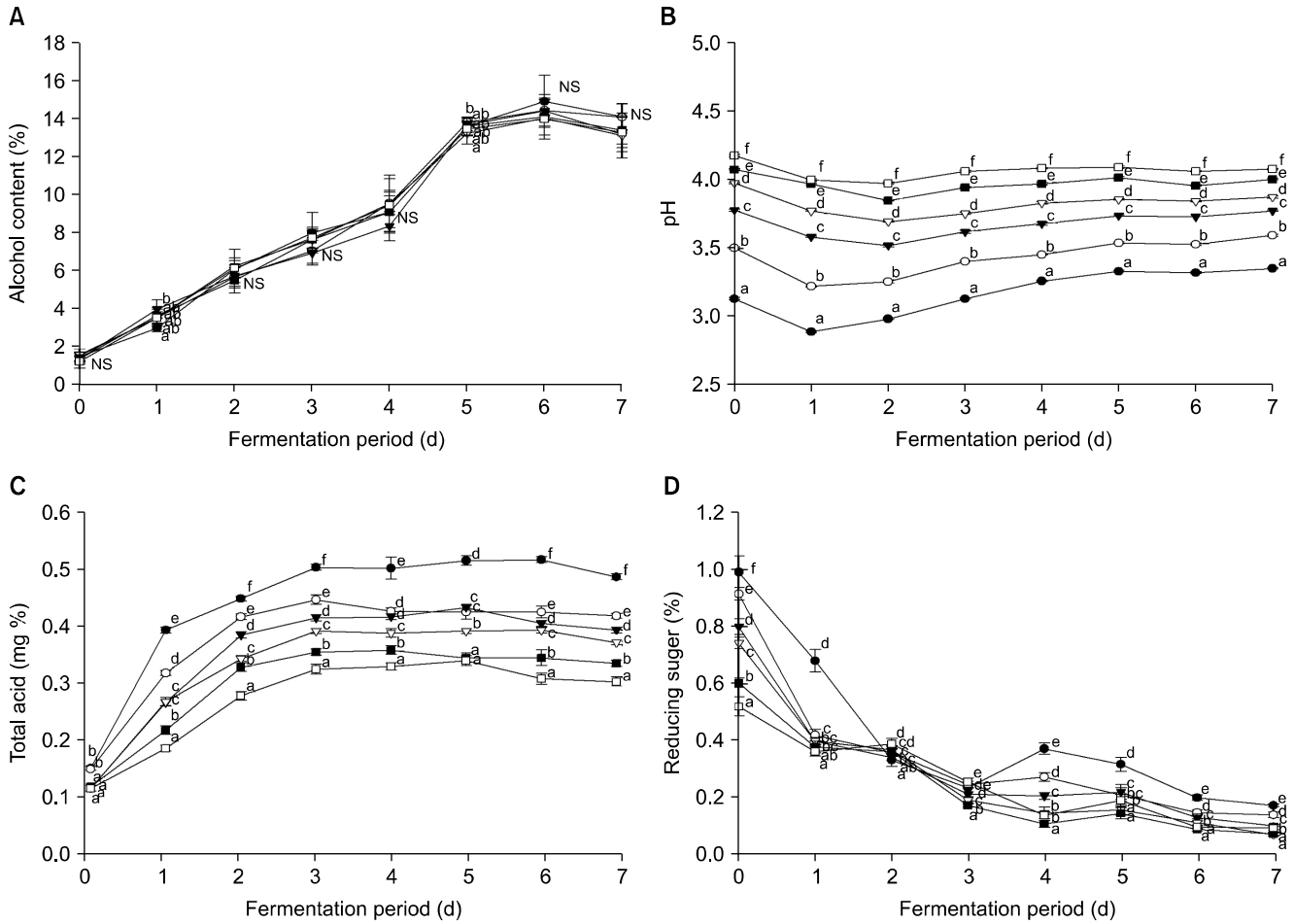

Fig. 1. Changes in (A) ethanol content, (B) pH, (C) total acid content, and (D) reducing sugar content during makgeolli fermentation in *L. japonica* makgeolli containing 0.0% (●), 2.5% (○), 5.0% (▼), 7.5% (▽), 10.0% (■), or 12.5% (□) of *L. japonica* powder. All measurements were performed in triplicate, and values are the means of three replicates. Means with different letters (a-f) among samples in the same fermentation period are significantly different at $P<0.05$ by Duncan's multiple range test.

The ethanol content peaked at 6 days after fermentation, regardless of the *L. japonica* concentration. After 7 days, the ethanol content decreased. A similar pattern was observed for blueberry makgeolli (11) and chestnut peel makgeolli (12).

The ethanol content of *L. japonica* makgeolli (>14.0%) was higher than that of ginseng makgeolli (5.9%) (9), blueberry makgeolli (12.9%) (11), and chestnut peel makgeolli (9.5%) (12), but lower than that of pear makgeolli (17.4%) (10) and cucumber makgeolli (16.2%) (13). The differences in the ethanol contents of these types of makgeolli were most likely a result of the use/non-use of mother brew and the extract of various substances from the plants during fermentation.

The ethanol content of *L. japonica* makgeolli was either unchanged or decreased slightly with increasing *L. japonica* concentration. This was likely due to the inhibition of alcohol fermentation by some unknown substances present in the *L. japonica* extract.

pH

Generally, the decrease in pH during makgeolli fermentation is the result of rapid organic acid production by lactic acid bacteria and yeast induced from the nuruk (7). This tendency was observed in previous studies of blueberry makgeolli (11) and chestnut peel makgeolli (12), which showed rapid decreases in pH within 2 days. However, makgeolli used koji (*Aspergillus niger* var. *niger* or *A. kawachii*) as a saccharifying agent, and the low pH was maintained by citric acid produced during fermentation.

In this study, to prepare *L. japonica* makgeolli, a two-step fermentation procedure with koji was adapted to enhance alcohol production. As shown in Fig. 1B, the 0-day results represent the time at which the second step was started. The pH of *L. japonica* makgeolli at time 0 was lower than that of other types of makgeolli using nuruk as a traditional saccharifying agent, perhaps due to the lower pH of the mother brew itself (3.8) and the citric acid produced by koji. From 1 to 2 days after fermentation, the pH of *L. japonica* makgeolli decreased due to citric acid produced by koji; after 2 or 3 days, the pH increased slightly.

The pH of *L. japonica* makgeolli increased from 3.0 to

4.2 in proportion with the increase in the concentration of *L. japonica* (0∼12.5%, w/w) at time 0. This may be due to the fact that the pH of the *L. japonica* water solution was 5.34 (data not shown).

Total acid content

The effects of *L. japonica* on total acid content during makgeolli fermentation were investigated (Fig. 1C). The total acid contents of all tested *L. japonica* makgeolli samples were less than 0.5 mg%. The total acid content of makgeolli that did not contain *L. japonica* was ≥0.5 mg% after 3 days. The total acid content decreased with increasing *L. japonica* concentration.

According to the Korean Food Standards Codex (34), if the total acid content of a makgeolli product is less than 0.5 mg %, the tested sample is regarded as normal. Generally, it is known that the lower the total acid content the better the quality of the makgeolli. In our study, the total acid contents of all tested *L. japonica* makgeolli samples were less than 0.5 mg %, indicating good quality in terms of total acid content.

Reducing sugars

In this study, the reducing sugar content was highest at time 0 (0.4∼1.0 mg %) (Fig. 1D). The reducing sugar content at time 0 decreased with increasing *L. japonica* concentration. After 7 days, the reducing sugar content was less than 0.2% in all tested makgeolli samples. The maximum reducing sugar contents of blueberry makgeolli (11) and chestnut peel makgeolli (12) were 14.0 and 7.5 mg %, respectively.

In general, during the early stages of makgeolli fermentation, the reducing sugar content increases dramatically due to the breakdown of starch into glucose by saccharifying enzymes produced by koji. Then, the glucose content decreases dramatically due to its conversion to ethanol by yeast. This tendency was also observed in this study.

Bacterial cell count

The initial low pH during makgeolli fermentation regulates bacterial growth. Up to 2 days after fermentation, the number of bacterial cells in *L. japonica* makgeolli increased from 3.3 to 3.9 log CFU/mL in proportion with the increase in *L. japonica* concentration. Thereafter, the bacterial cell density decreased irrespective of the *L. japonica* concentration, likely due to growth inhibition by the ethanol produced during fermentation (Fig. 2A).

In general, the number of bacterial cells in manufactured makgeolli was higher when nuruk was used as a starch-degrading enzyme source during makgeolli production than when koji was used. In the present study, the number of bacterial cells in makgeolli containing no *L. japonica* and prepared using koji was ∼2.0 log CFU/mL, which was lower than for blueberry makgeolli (9.5 log CFU/mL) (11) and chestnut peel makgeolli (8.0 log CFU/mL) (12) at 2 days after fermentation. The dominant bacteria of blueberry and chestnut peel makgeolli were considered to be lactic acid bacteria and acid-resistant bacteria originating from nuruk. Otherwise, the microorganisms in makgeolli produced in this study were not considered to be lactic acid bacteria, but acid-resistant bacteria.

Yeast counts

The number of yeast cells at the initial stage of *L. japonica* makgeolli fermentation was 8.0 log CFU/mL, which is relatively high. After 2∼3 days, the number of yeast cells reached 9.2∼9.4 log CFU/mL, the maximum detected. In previous reports of blueberry makgeolli (11) and chestnut peel makgeolli (12), the maximum numbers of yeast cells were 8.5 and 8.0 log CFU/g, respec-

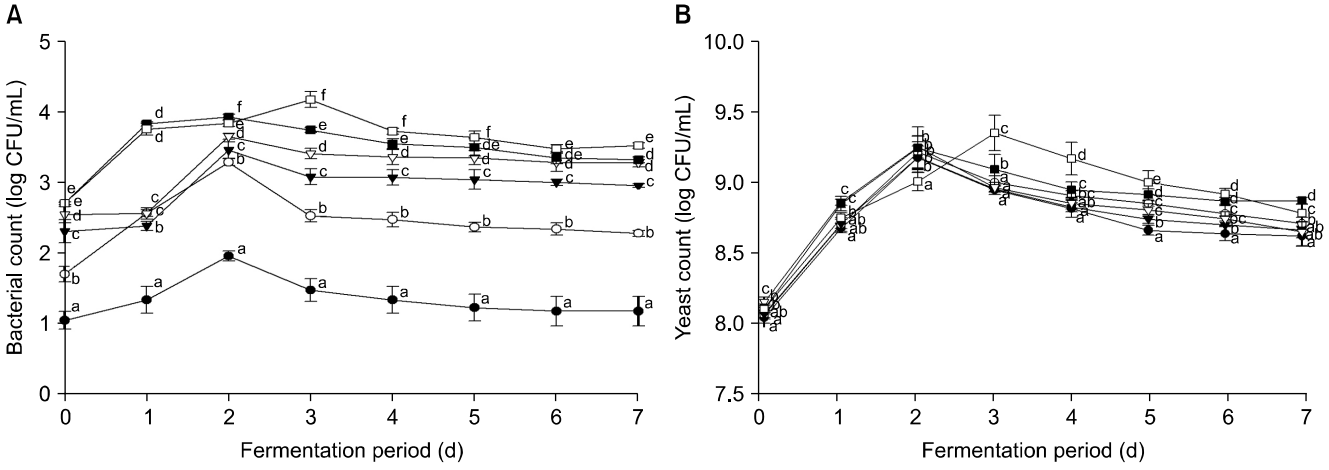

Fig. 2. Changes in (A) bacterial count, and (B) yeast count during makgeolli fermentation in *L. japonica* makgeolli containing 0.0% (●), 2.5% (○), 5.0% (▼), 7.5% (▽), 10.0% (■), or 12.5% (□) of *L. japonica* powder. All measurements were performed in triplicate, and values are the means of three replicates. Means with different letters (a–f) among samples in the same fermentation period are significantly different at $P<0.05$ by Duncan's multiple range test.

tively. The greatest increase in yeast number occurred at 2～3 days after fermentation. After 2～3 days, the number of yeast decreased along with the consumption of reducing sugar. This may be due to the transient increase in reducing sugars at 4～5 days after fermentation. The relatively high number of yeast cells in our study was likely caused by the use of a two-step fermentation process (Fig. 2B).

Free sugars

To determine the change in free sugar content during fermentation, assays for fructose, glucose, and sucrose were conducted (Table 1). Free sugars other than glucose were not detected in any of the tested groups (data not shown). In makgeolli without *L. japonica*, all glucose was consumed within 2 days. In makgeolli containing *L. japonica*, the glucose content declined gradually during the fermentation period, but remained detectable at 7 days in all samples tested. The glucose content at 7 days increased slightly and proportionally with the amount of *L. japonica*.

Organic acids

To determine the changes in organic acid content during fermentation, assays for acetic acid, citric acid, succinic acid, and lactic acid were conducted (Table 2). The contents of lactic acid, citric acid, and succinic acid, but not acetic acid, increased slowly during the fermentation process, regardless of the quantity of *L. japonica* added. The acetic acid content was increased at 2 days after fer-

Table 1. Changes in glucose contents during fermentation (Units: %)

Sample	Fermentation period (d)							
	0	1	2	3	4	5	6	7
Control	0.52±0.10^{a}	0.43±0.08^{a}	0.38±0.08^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
2.5% *L. japonica*	0.77±0.20^{b}	0.63±0.08^{b}	0.48±0.08^{b}	0.28±0.04^{b}	0.18±0.10^{b}	0.08±0.04^{b}	0.20±0.00^{b}	0.20±0.00^{b}
5% *L. japonica*	0.52±0.13^{a}	0.65±0.18^{bc}	0.63±0.08^{c}	0.50±0.00^{c}	0.50±0.00^{c}	0.38±0.08^{c}	0.38±0.04^{c}	0.35±0.06^{c}
7.5% *L. japonica*	1.08±0.04^{c}	0.77±0.05^{cd}	0.70±0.05^{c}	0.67±0.05^{d}	0.60±0.00^{d}	0.53±0.08^{d}	0.50±0.00^{d}	0.45±0.06^{d}
10% *L. japonica*	1.22±0.04^{d}	0.85±0.06^{d}	0.88±0.04^{d}	0.90±0.00^{e}	0.80±0.11^{e}	0.75±0.08^{e}	0.58±0.04^{e}	0.50±0.00^{e}
12.5% *L. japonica*	1.50±0.06^{e}	1.37±0.10^{e}	1.12±0.15^{e}	1.17±0.08^{f}	0.98±0.04^{f}	1.03±0.08^{f}	0.70±0.00^{f}	0.70±0.00^{f}

All measurements were performed in triplicate, and values are the means of three replicates.
$^{a-f}$Means with different superscripts in the same column are significantly different at $P<0.05$ by Duncan's multiple range test.

Table 2. Changes in acetic acid, citric acid, succinic acid, and lactic acid contents during fermentation (Units: mg%)

Organic acid contents (mg%)	Sample	Fermentation period (d)							
		0	1	2	3	4	5	6	7
Acetic acid	Control	3.32±0.13^{dA}	15.74±0.16^{fD}	12.72±0.84^{cC}	14.55±1.10^{cCD}	10.14±1.48^{cB}	12.38±4.97^{nsBC}	15.90±0.75^{aD}	14.78±0.72^{aCD}
	2.5% *L. japonica*	2.77±0.23^{cA}	11.72±0.19^{eC}	11.24±0.79^{bC}	7.46±1.14^{bB}	5.97±1.36^{bB}	10.45±5.36^{C}	10.24±0.54^{bC}	11.18±0.76^{bC}
	5% *L. japonica*	2.52±0.14^{bA}	10.15±0.20^{dC}	11.71±0.91^{bcCD}	7.331±1.21^{bBC}	4.96±0.24^{abAB}	15.37±10.78^{DE}	16.58±0.56^{cE}	17.34±1.17^{cE}
	7.5% *L. japonica*	2.18±0.12^{aA}	8.39±0.34^{cAB}	10.38±1.18^{bB}	7.47±0.54^{bAB}	5.47±0.21^{abAB}	21.03±16.18^{C}	21.95±0.63^{dC}	23.22±0.90^{dC}
	10% *L. japonica*	2.04±0.19^{aA}	5.18±0.36^{bA}	8.78±1.28^{aA}	5.40±0.26^{aA}	4.81±0.24^{aA}	25.03±21.96^{B}	28.04±0.97^{eB}	29.75±0.45^{eB}
	12.5% *L. japonica*	1.98±0.11^{aA}	3.81±0.25^{aA}	8.54±1.49^{aA}	4.90±0.18^{aA}	4.47±0.24^{aA}	29.48±27.44^{B}	33.08±1.20^{fB}	34.69±0.38^{fB}
Citric acid	Control	1.43±0.14^{cA}	1.99±0.24^{cA}	2.21±0.14^{cA}	5.21±1.00^{cB}	5.99±0.80^{eBC}	6.75±2.61^{cC}	5.23±0.13^{eB}	5.72±0.31^{fBC}
	2.5% *L. japonica*	0.96±0.13^{bA}	0.65±0.09^{abA}	1.51±0.16^{cA}	3.18±0.52^{cB}	3.64±0.33^{dB}	6.91±2.22^{bC}	8.34±0.66^{dD}	8.53±0.67^{eD}
	5% *L. japonica*	0.77±0.07^{aA}	0.78±0.24^{abA}	1.59±0.16^{bA}	2.89±0.37^{bB}	3.13±0.13^{cB}	6.39±1.95^{abC}	7.56±0.61^{cD}	7.15±0.38^{dCD}
	7.5% *L. japonica*	0.94±0.04^{bA}	0.94±0.35^{bA}	1.53±0.09^{bA}	2.81±0.12^{bB}	2.76±0.18^{cB}	6.26±2.10^{abCD}	7.07±0.48^{cD}	5.91±0.58^{cC}
	10% *L. japonica*	0.96±0.05^{bA}	0.89±0.42^{bA}	1.07±0.07^{aA}	1.88±0.08^{aB}	1.95±0.08^{bB}	4.07±0.86^{abC}	4.58±0.37^{bC}	4.56±0.58^{bC}
	12.5% *L. japonica*	0.95±0.08^{bAB}	0.54±0.09^{aA}	1.07±0.09^{aBC}	1.38±0.09^{aC}	1.45±0.17^{aC}	3.32±0.83^{aD}	3.28±0.29^{aD}	3.69±0.41^{aD}
Succinic acid	Control	1.63±0.11^{aA}	2.12±0.11^{abA}	2.05±0.16^{aA}	2.66±0.19^{aB}	2.67±0.11^{bB}	4.02±0.48^{cC}	4.47±0.62^{bC}	4.32±0.74^{bC}
	2.5% *L. japonica*	1.72±0.06^{bcA}	1.92±0.23^{aAB}	2.20±0.30^{abB}	2.99±0.21^{cD}	2.57±0.23^{abC}	3.50±0.05^{abE}	3.44±0.39^{aE}	3.40±0.52^{aE}
	5% *L. japonica*	1.61±0.07^{aA}	2.13±0.28^{abB}	2.22±0.26^{abBC}	2.82±0.09^{abcD}	2.46±0.12^{aC}	3.66±0.17^{abE}	3.54±0.35^{aE}	3.56±0.34^{aE}
	7.5% *L. japonica*	1.61±0.04^{aA}	2.12±0.16^{abB}	2.37±0.10^{bBC}	2.87±0.10^{bcD}	2.51±0.16^{abC}	3.74±0.19^{bcF}	3.60±0.27^{aF}	3.30±0.54^{aE}
	10% *L. japonica*	1.71±0.03^{abA}	2.17±0.07^{bB}	2.17±0.07^{abB}	2.73±0.14^{abC}	2.58±0.09^{abC}	3.44±0.20^{abE}	3.63±0.14^{aE}	3.10±0.62^{aD}
	12.5% *L. japonica*	1.79±0.02^{cA}	2.14±0.07^{bB}	2.05±0.06^{aAB}	2.74±0.25^{abCD}	2.56±0.09^{abC}	3.32±0.26^{aE}	3.54±0.23^{aE}	2.95±0.54^{aD}
Lactic acid	Control	2.72±0.30^{bcA}	2.84±0.33^{abA}	3.56±0.54^{aB}	5.02±0.37^{aC}	5.95±0.97^{nsD}	6.00±0.13^{nsD}	5.67±0.24^{aD}	6.15±0.45^{nsD}
	2.5% *L. japonica*	2.60±0.16^{abA}	2.42±0.32^{aA}	4.16±0.12^{bB}	5.05±0.14^{abC}	5.57±0.96^{C}	5.43±0.73^{C}	5.58±0.46^{aC}	6.45±0.92^{D}
	5% *L. japonica*	2.43±0.11^{aA}	2.83±0.16^{abA}	4.24±0.52^{bB}	5.39±0.15^{bcC}	5.79±0.83^{CD}	5.91±0.91^{CD}	6.48±0.38^{bD}	6.54±0.68^{D}
	7.5% *L. japonica*	2.47±0.12^{aA}	3.14±0.17^{bcB}	4.94±0.19^{cC}	5.63±0.14^{cdD}	6.21±0.81^{D}	6.20±0.82^{D}	7.29±0.15^{cE}	6.08±0.90^{D}
	10% *L. japonica*	2.88±0.17^{cA}	3.44±0.51^{cA}	4.76±0.09^{cB}	5.59±0.37^{cC}	6.35±0.34^{D}	5.87±0.50^{CD}	7.54±0.36^{cE}	5.93±1.05^{CD}
	12.5% *L. japonica*	3.21±0.20^{dA}	3.52±0.43^{cA}	4.79±0.29^{cB}	5.98±0.53^{dC}	6.30±0.25^{C}	6.07±0.44^{C}	7.28±0.33^{cD}	6.28±0.77^{C}

All measurements were performed in triplicate, and values are the means of three replicates.
ns: not significant.
$^{a-f}$Means with different superscripts within the same column of each organic acid are significantly different at $P<0.05$ by Duncan's multiple range test.
$^{A-E}$Means with different superscripts in the same row are significantly different at $P<0.05$ by Duncan's multiple range test.

mentation, decreased at 4 days, and increased dramatically and proportionally with the quantity of *L. japonica* at 7 days.

According to a previous report of the relationship between organic acids and the sourness of makgeolli, lactic acid is the major organic acid present in makgeolli; other organic acids, such as acetic acid, malic acid, propionic acid, are present at levels of less than 10% of the lactic acid content (35). In *L. japonica* makgeolli, the major organic acid is acetic acid; citric acid, lactic acid, and succinic acid are also present (in that order of contents). These results are not congruous with the reports by Choi et al. (36) and Lee et al. (35), in that despite differences according to the fermentation process and the ratio of raw materials, the lactic acid content is far higher than that of other organic acids.

In general, in makgeolli produced using nuruk, the lactic acid content increases 10-fold compared to that at the beginning of the fermentation process due to the presence of lactic acid bacteria in nuruk. When koji is used, as in our study, the lactic acid content increases slightly. However, in this study, the major organic acid in *L. japonica* makgeolli was acetic acid, likely due to the effect on makgeolli fermentation of some unknown substances in the *L. japonica* extracts.

Color value

The color of *L. japonica* makgeolli was determined using a color difference meter (CM-700d, Minolta) and is described as the *L* value (lightness), *a* value (redness), and *b* value (yellowness). The *L*, *a*, and *b* values of makgeolli without *L. japonica* were 43.55, 0.14, and 4.97, respectively. The color was a bright yellowish white.

In makgeolli containing 12.5% *L. japonica*, the *L*, *a*, and *b* values were 31.28, 0.06, and 6.12, respectively. Compared to makgeolli without *L. japonica*, the *L* value of makgeolli containing 12.5% *L. japonica* was lower, the *b* value was slightly higher, and the *a* value was similar. A larger overall color difference (ΔE) represents a greater color change. The ΔE value of makgeolli became higher with addition of increasing quantities of *L. japonica* (Table 3). The *L. japonica* makgeolli was a yellowish olive-green color.

Acceptability of *L. japonica* makgeolli

The effects of the quantity of *L. japonica* added on the acceptability of *L. japonica* makgeolli were investigated (Table 4). As the quantity added increased, the bitterness and seaweed flavor increased, whereas kojic flavors, sweetness, and refreshment decreased. Sourness was uniformly low among the *L. japonica* makgeolli samples.

From this sensory evaluation, the *L. japonica* makgeolli brewed by adding 2.5% or 5% *L. japonica* to the mash showed the best overall acceptability, which decreased in the following order: no makgeolli, 7.5% *L. japonica* makgeolli, 10% *L. japonica* makgeolli, and 12.5% *L. japon-*

Table 3. Color change of *L. japonica* makgeolli

L. japonica (%)	Hunter's color value			
	L (lightness)	*a* (redness)	*b* (yellowness)	ΔE[1)]
0	43.55±1.33^{d}	0.14±0.08^{ab}	4.97±0.07^{a}	–
2.5	39.11±2.04^{c}	−0.41±0.09^{a}	6.15±0.51^{c}	4.63
5	33.18±1.16^{b}	−0.21±0.10^{ab}	5.49±0.37^{b}	10.39
7.5	32.83±1.00^{ab}	−0.10±0.07^{ab}	5.89±0.25^{c}	10.76
10	31.94±0.81^{ab}	−0.02±0.08^{ab}	6.12±0.20^{c}	11.67
12.5	31.28±0.97^{a}	0.06±0.06^{b}	6.12±0.38^{c}	12.32

[1)] ΔE: Overall color difference $[(\Delta L)^2+(\Delta a)^2+(\Delta b)^2]^{1/2}$.
All measurements were performed in triplicate, and values are the means of three replicates.
[a–d]Means with different superscripts in the same column are significantly different at $P<0.05$ by Duncan's multiple range test.

Table 4. Sensory evaluation of fresh makgeolli

	L. japonica (%)					
	0	2.5	5	7.5	10	12.5
Flavor	7.14±1.46^{b}	7.0±1.60^{b}	6.71±1.48^{b}	6.14±1.64^{b}	4.00±1.41^{a}	3.14±1.13^{a}
Seaweed flavor	1.00±0.00^{a}	1.17±0.37^{a}	2.00±1.41^{ab}	3.17±2.03^{bc}	4.50±1.71^{cd}	6.00±1.63^{d}
Sweetness	6.14±2.10^{b}	5.14±1.55^{ab}	5.29±1.39^{ab}	5.00±0.93^{ab}	4.71±1.03^{ab}	4.00±1.31^{a}
Bitterness	2.14±0.99^{ns}	2.86±1.96	3.29±1.67	3.00±1.41	3.57±1.50	4.14±2.10
Sourness	2.57±1.60^{ns}	2.71±1.67	2.43±1.29	2.43±1.29	2.57±1.18	2.71±1.16
Refreshment	7.50±1.50^{cd}	8.00±1.31^{d}	7.80±0.64^{cd}	6.57±1.29^{bc}	5.50±0.90^{ab}	4.71±0.88^{a}
Overall acceptability	7.43±1.68^{b}	8.14±1.36^{b}	8.14±0.83^{b}	6.71±1.39^{b}	5.14±0.99^{a}	4.71±1.28^{a}

All measurements were performed in triplicate, and values are the means of three replicates.
ns: not significant.
[a–d]Means with different superscripts in the same row are significantly different at $P<0.05$ by Duncan's multiple range test.

ica makgeolli. Notably, 12.5% *L. japonica* makgeolli provoked aversion due to its seaweed taste. In the case of makgeolli containing <5% *L. japonica*, almost no seaweed flavor was detected. This was most likely a result of reductions in the levels of the materials responsible for the seaweed flavor during fermentation. Such a tendency was also reported by Seo et al. (37), who demonstrated removal of off-flavors from *L. japonica* extract by fermentation with *Aspergillus oryzae*.

A sensory evaluation of *L. japonica* makgeolli containing no added sweetener (e.g., aspartame) was also conducted. For commercial purposes, when we added 0.125% aspartame to *L. japonica* makgeolli and then tested its overall acceptability, 2.5~7.5% *L. japonica* makgeolli showed the best overall acceptability, and did not have a seaweed flavor (data not shown).

Peroxynitrite-scavenging activity of *L. japonica* makgeolli

The peroxynitrite ($ONOO^-$)-scavenging activity (antioxidant activity) of *L. japonica* makgeolli was 0.73~0.90 times that of control makgeolli (no added *L. japonica*; 66.29%) (Fig. 3A). The IC_{50} values of *L. japonica* makgeolli extracts were 79~110 μg/mL. The IC_{50} values of control makgeolli and penicillamine (a positive control) were 70 and 4.33 μg/mL, respectively. The antioxidant activity of *L. japonica* makgeolli extract is lower than that of makgeolli with no added *L. japonica*. *L. japonica* has antioxidant activity (18,19), but adding *L. japonica* to makgeolli had no effect on the antioxidant activity. Based on previous reports (38,39), the antioxidant activities of the *L. japonica* crude extract itself may not be very high. Indeed, the antioxidant activities of the *L. japonica* hot water extract, *L. japonica* ethanol extract, and *L. japonica* methanol extract were reportedly <10% at a concentration of 1 mg/mL (38). Therefore, because the antioxidant activity of makgeolli itself was significantly superior to that of *L. japonica* extract, adding *L. japonica* to makgeolli may not have affected the antioxidant activity. Alternatively, known or unknown antioxidant compounds in *L. japonica* might have been degraded during fermentation. Further research is needed to elucidate the cause.

Inhibition of ACE activity

No ACE inhibition was observed in any of the makgeolli samples. *L. japonica* has been reported to have anti-hypertensive effects (24). Thus, we examined the ACE inhibitory activity of *L. japonica* makgeolli. In this report, no ACE inhibitory activities of *L. japonica* makgeolli were detected. Captopril (1 μg/mL) produced 51.17±0.05% inhibition of ACE activity (data not shown). In previous studies, the ACE-inhibitory effect of *L. japonica* hot water extract and *L. japonica* methanol extract varied from 7% to 73% (40,41). According to Cha et al. (40), the ACE-inhibitory activity of the enzyme-hydrolyzed seaweed extract decreased considerably at 12 h after the enzyme-digestion reaction. In this study, no ACE-inhibitory activity of *L. japonica* makgeolli was detected, likely because of degradation of active compounds during fermentation. This tendency is similar to that reported previously.

Anti-diabetes activity

The PTP1B inhibitory effects of supernatants of makgeolli containing 0.0, 2.5, 5.0, 7.5, 10.0, and 12.5% *L. japonica* were 26.79, 28.50, 57.19, 102.54, 100.19, and 99.24%, respectively (Fig. 3B). Notably, the supernatants of *L. japonica* makgeolli extracts containing 7.5~12.5% *L. japonica* showed potent inhibitory effects (99.24~102.54%). The IC_{50} values of supernatants of makgeolli containing 5.0, 7.5, 10.0, and 12.5% *L. japonica* were 22.21, 0.84, 0.22, and 0.11 μg/mL, respectively. The IC_{50} value of a hot-water extract of dried *L. japonica* powder was 0.410 μg/mL. On the basis of these results, *L. japonica* makgeolli showed potent anti-diabetes activity, partic-

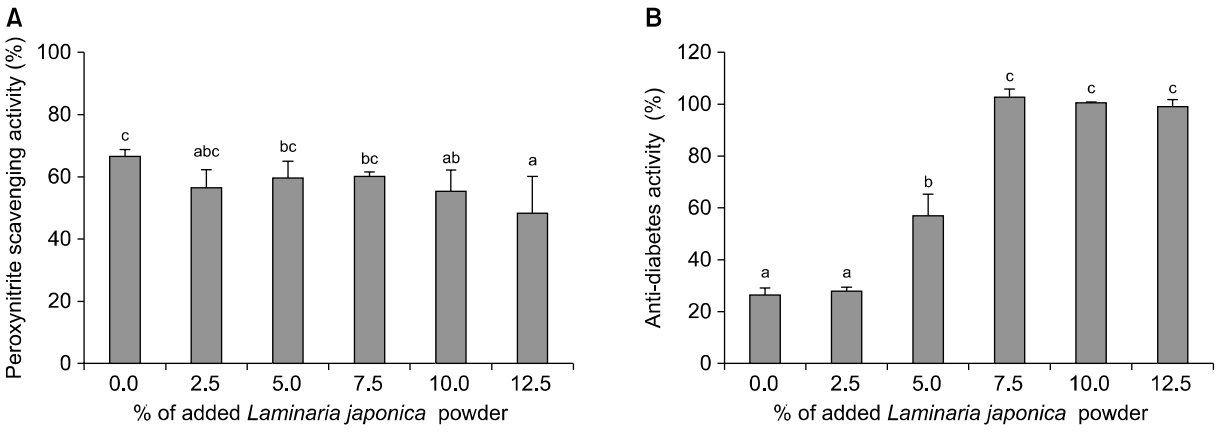

Fig. 3. Peroxynitrite-scavenging activity (A) and anti-diabetes activity (B) of *L. japonica* makgeolli. All measurements were performed in triplicate, and values are means of three replicates. All measurements were performed in triplicate, and values are the means of three replicates. Means with different letters (a-c) above the bars are significantly different at $P<0.05$ by Duncan's multiple range test.

ularly *L. japonica* makgeolli containing >7.5% *L. japonica*.

In conclusion, *L. japonica* makgeolli containing 5.0 or 7.5% *L. japonica* exhibited considerable anti-diabetes activity and showed the best overall acceptability, with no seaweed flavor.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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