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Research Article

Metabolite Profiling during Fermentation of *Makgeolli* by the Wild Yeast Strain *Saccharomyces cerevisiae* Y98-5

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Abstract *Makgeolli* is a traditional Korean alcoholic beverage. The flavor of *makgeolli* is primarily determined by metabolic products such as free sugars, amino acids, organic acids, and aromatic compounds, which are produced during the fermentation of raw materials by molds and yeasts present in *nuruk*, a Korean fermentation starter. In this study, *makgeolli* was brewed using the wild yeast strain *Saccharomyces cerevisiae* Y98-5, and temporal changes in the metabolites during fermentation were analyzed by ultra-high-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. The resultant data were analyzed by partial least squares-discriminant analysis (PLS-DA). Various metabolites, including amino acids, organic acids, sugar alcohols, small peptides, and nucleosides, were obviously altered by increasing the fermentation period. Changes in these metabolites allowed us to distinguish among *makgeolli* samples with different fermentation periods (1, 2, 3, 6, 7, and 8 days) on a PLS-DA score plot. In the *makgeolli* brewed in this study, the amounts of tyrosine (463.13 μg/mL) and leucine (362.77 μg/mL) were high. Therefore, our results indicate that monitoring the changes in metabolites during *makgeolli* fermentation might be important for brewing *makgeolli* with good nutritional quality.

Keywords Fermentation, *Makgeolli*, Metabolite, *Saccharomyces cerevisiae*

Makgeolli is a traditional Korean alcoholic beverage. It is brewed from rice and *nuruk* (a Korean fermentation starter) and roughly filtered before serving. *Makgeolli* is mainly consumed by the general public [1]. In the case of *makgeolli*, the entire fermented material is homogenized and consumed as it stands, unlike alcoholic beverages that are more finely filtered (e.g., *cheongju* or *yakju*). Thus, *makgeolli* includes the vitamin B group, essential amino acids, glutathione, as well as proteins, oligosaccharides, and live yeast. Accordingly, it has nutritional characteristics that are different from those of other alcoholic beverages [2]. With the recent increase in the consumption of *makgeolli*, studies on the functional effects and flavor components of *makgeolli* have also increased. For example, it has been reported that *makgeolli* has anticancer effects [3, 4], effects on blood circulation and lipids [5], antihypertensive activity [6, 7], fibrinolytic and superoxide dismutase-like activity [8], and antibacterial/antioxidant activity [9]. Studies on the volatile flavor components of *takju* (a type of *makgeolli*) have shown that they depend on the type of yeast [10] and the raw material [11]. In addition, many studies have focused on the strains used for *makgeolli* fermentation. These studies include those conducted for the selection of *koji* (*Aspergillus* spp.) and yeast for the improvement of fermentation characteristics and *cheongju* quality [12]; isolation and identification of a yeast strain that produces abundant glutathione (a biologically active substance) and determination of the optimal production conditions [13]; screening of brewing yeasts and saccharifying molds for foxtail millet wine-making and examination of the brewing characteristics of the selected stains [14, 15]; determination of changes in microflora during fermentation of *takju* and *yakju* [16]; isolation and identification of yeast strains with high viability that produce a high concentration of ethanol [17]; isolation and characterization of ethanol-tolerant yeast [18]; and finally, research on the production of biologically active substances such as an antihypertensive angiotensin-

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converting-enzyme inhibitor [19] and an antidementia β-secretase inhibitor [20] from *Saccharomyces cerevisiae*. However, there has been no analysis of the metabolite profile during the fermentation of *makgeolli*.

Therefore, the aim of this study was to analyze changes in the metabolite profile during fermentation of *makgeolli* brewed with *koji* and yeast isolated from traditional Korean *nuruk*.

MATERIALS AND METHODS

Strains and chemicals. Yeasts isolated from *nuruk* were used in this study. *Saccharomyces cerevisiae* Y98-5 was collected from the Gongju area of Chungnam province [21]. *Koji* (saccharogenic power [sp] 85) was purchased from Seoul Jangsoo, Inc. (Jincheon, Korea). The amino acids standard and organic acids were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All reagents used for ultra-high-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF MS) analyses were of high-performance liquid chromatography grade.

***Makgeolli* brewing.** The first brewing (yeast, 0.02% and *koji* [sp 85] : distilled water = 38 : 62) was performed to reach 36% of the total *makgeolli* volume and was followed by fermentation at 25°C for 2 days. The second brewing (steamed non-glutinous rice : water = 32 : 68; 64% of the total *makgeolli* volume) was then performed, followed by fermentation at 25°C for 8 days. After compression, *makgeolli* was prepared by filtration through a 120-mesh filter. *Makgeolli* brewing was performed in triplicate.

Chemical analysis. The concentration of soluble solids was measured with a handheld refractometer (ATAGO Pocket PAL-1; ATAGO Co. Ltd., Tokyo, Japan) and recorded in Brix units (% sucrose). The pH was measured with a model D-51 pH meter (HORIBA, Kyoto, Japan).

Metabolite extraction. To extract metabolites for UHPLC-Q-TOF MS analysis, 0.9 mL of 50% MeOH (internal standard reserpine, 10 ppm) was added to 0.1 mL of *makgeolli*; after vortexing for 5 min, the mixture was kept at 4°C for 16 hr. Next, centrifugation was performed at 14,000 rpm at 4°C for 20 min; the supernatant was then collected and the metabolites were extracted.

Metabolomic analysis. For analysis of the metabolome, we used an Agilent (Santa Clara, CA, USA) UHPLC-Q-TOF MS system (UHPLC, Agilent 1290 Infinity; MS, Agilent 6520 with Jet Stream Technology) controlled by MassHunter Workstation Data Acquisition software v. B. 05.00 (Agilent). Using the ESI + Jet Stream method, in the positive ionization mode, the gas temperature was set at 325°C, the drying gas (N_2) flow at 8 L/mL, the nebulizer pressure at 30 psi, the capillary voltage at 4,000 V, the skimmer voltage at 65 V, and the fragmentor voltage at 70 V. In the negative ionization mode, the gas temperature was set at 325°C, the drying gas flow at 8 L/mL, the nebulizer pressure at 30 psi, the capillary voltage at 3,500 V, the skimmer voltage at 65 V, and the fragmentor voltage at 50 V. For the mobile phase of UHPLC, a gradient of 5 mM ammonium acetate in water (A) and 0.1% formic acid in acetonitrile (B) was used. Using a ZORBAX HILIC Plus (2.1 × 100 mm, 3.5 μm; Agilent) column, the analysis was performed at a flow rate of 0.3 mL/min and a column temperature of 30°C. The data were aligned and normalized using Mass Profiler Professional (Agilent), and multivariate statistical analysis was performed using SIMCA-P+ 12.0.1 (Umetrics, Umea, Sweden).

RESULTS AND DISCUSSION

Changes in chemical properties during fermentation. *Makgeolli* was brewed using *S. cerevisiae* Y98-5 (isolated from *nuruk*) and *koji* as fermenting agents. *Koji* consists of non-glutinous rice inoculated with *Aspergillus* species. Fig. 1 shows the changes in the soluble solids content and pH during fermentation of the *makgeolli*. The pH was 3.08 on the first day of fermentation and then gradually increased, reaching 3.7 upon the completion of fermentation. The pH was similar to that of *makgeolli* brewed using *koji* made from different rice varieties, as reported in Kwon *et al.* [22]. Furthermore, it was similar to the pH of *nuruk* mash prepared using *Aspergillus oryzae* and *Aspergillus kawachii*, as reported in Han *et al.* [23].

The soluble solids content was 4.3% during the early stage of fermentation. It then increased, reaching a maximum value (10.6%) on the seventh day of fermentation, before decreasing to 10.0% upon the completion of fermentation. The soluble solids content reflects the amount of sugar remaining after two processes: amylolysis of rice starch by the *koji* mold at the early stage of fermentation, and use of the resulting sugar as a carbon source by *S. cerevisiae* Y98-5 for propagation and alcohol fermentation (final ethanol content was 15%). In the case of the *makgeolli* brewed with

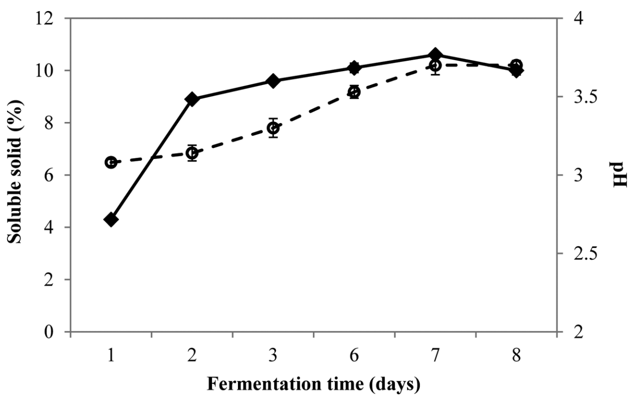

Fig. 1. Changes of soluble solids content (◆) and pH (○) during fermentation of *makgeolli* brewed with *Saccharomyces cerevisiae* Y98-5. Each data point represents the mean ± SD (n = 3).

S. cerevisiae Y98-5 and *koji* as fermenting agents, abnormal fermentation did not occur. The pH was 3.7 and the soluble solids content was 10% upon the completion of fermentation. During the making of *makgeolli* by dilution with water after the completion of fermentation, a pH and soluble solids content suitable for drinking were maintained.

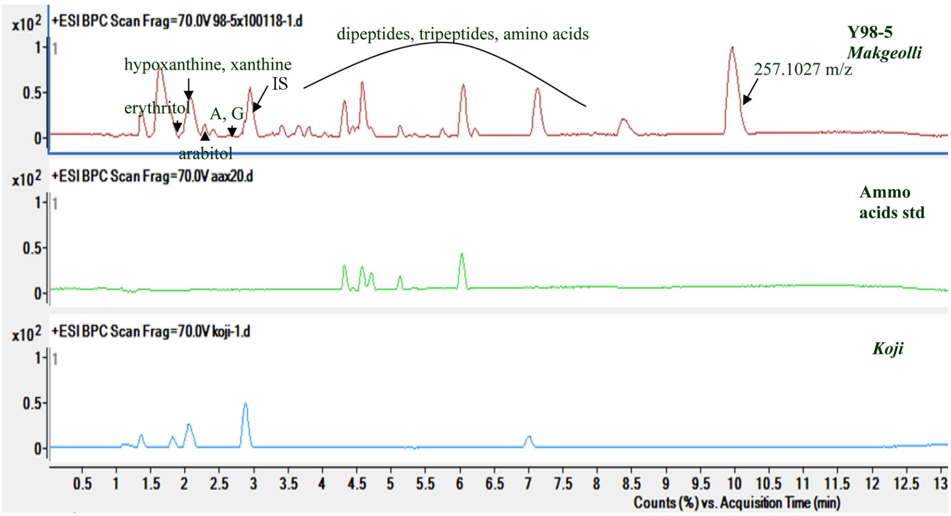

Fig. 2. Total ion chromatograms of *makgeolli* brewed with *Saccharomyces cerevisiae* Y98-5, an amino acids standard, and *koji*. IS, internal standard; A, adenine; G, guanine.

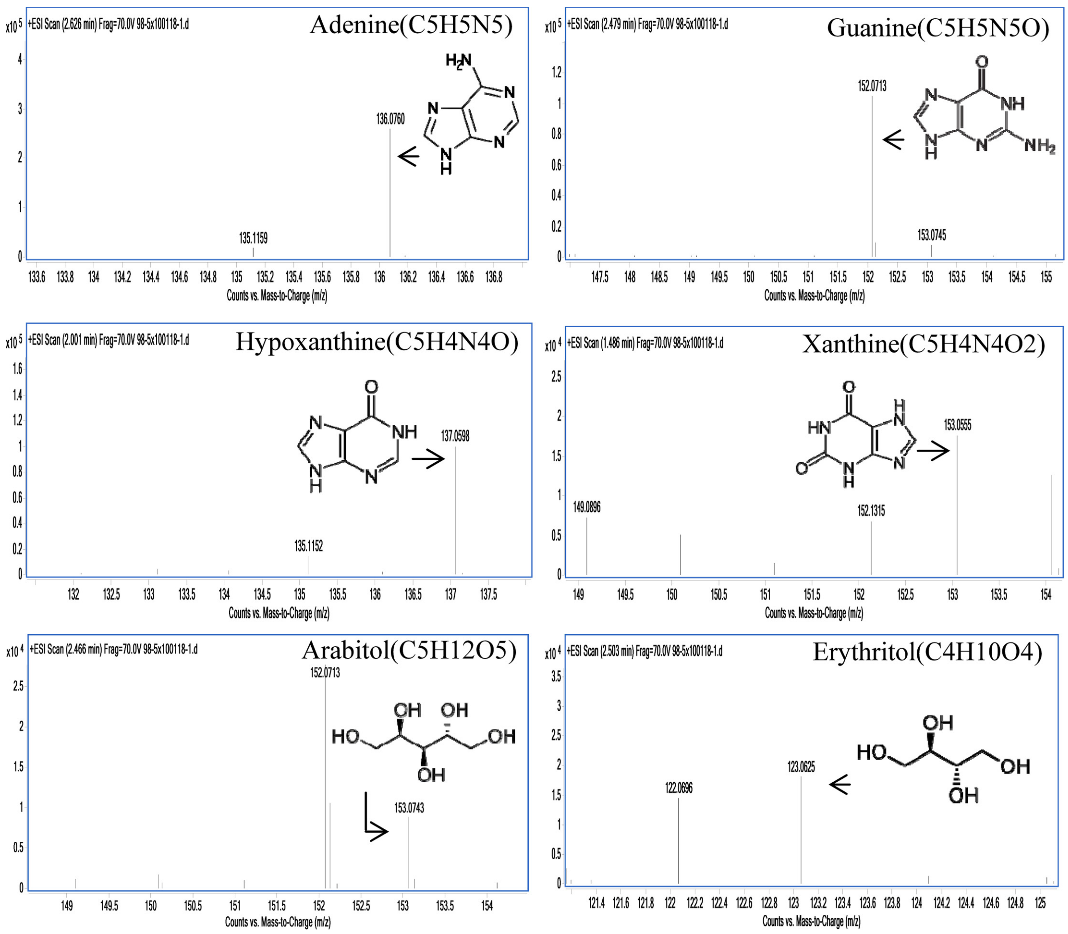

Fig. 3. Electron ionization mass spectra of $[M + H]^+$ ions of adenine, guanine, hypoxanthine, xanthine, arabitol, and erythritol at a collision energy of 70 eV.

Metabolomic profiling of *makgeolli* during fermentation. The metabolome of the *S. cerevisiae* Y98-5 *makgeolli* during fermentation was analyzed using UHPLC-Q-TOF MS, and 296 metabolites were detected. Most metabolites had a mass value less than 800. Fig. 2 shows the total ion chromatogram (TIC) for the metabolome on the eighth day of fermentation as well as TICs for the *koji* and amino acids standard. The TIC for the *makgeolli* on the eighth day of fermentation was broadly divided into peaks between 1 and 2.5 min, a peak at 2.9 min (internal standard), peaks between 3.5 and 6.5 min, a peak at 7.2 min, and a peak at 9.87 min. The peaks between 1 and 2.5 min were identified as adenine, guanine, hypoxanthine, and xanthine, which originated from the yeast cells inoculated during the brewing of *makgeolli* and the fungus in the *koji*, and as arabitol and erythritol, which are sugar alcohols (Fig. 3). These peaks increased in the *makgeolli* TIC compared with the *koji* TIC. The peaks between 3.5 and 6.5 min were identified as dipeptides, such as Ser-Val and Glu-Val, and tripeptides, such as Phe-Arg-Asn and Val-Arg-Val (Fig. 4). The pattern of peaks between 4.2 and 8.2 min was similar to the peak pattern of the amino acids standard. The results indicated the presence of 16 amino acids and the nonprotein amino acid γ-amino-*n*-butyric acid (GABA). The peak observed for the *makgeolli* at 9.87 min was due to fermentation and was attributed to an $[M + H]^+$ ion at *m/z* 257.1027.

A partial least squares-discriminant analysis (PLS-DA) of the metabolome of Y98-5 *makgeolli* during the fermentation period was performed using SIMCA-P+. As shown in Fig. 5, the *makgeolli* samples taken at different fermentation times were clearly distinguishable in the score plot generated by combining PC1 (30.15% of the total variance) with PC2 (18.40% of the total variance). Based on PC1, the first-, second-, and third-day fermentation samples were positioned on the right side of the plot and the sixth-, seventh-, and eighth-day fermentation samples were positioned on the left side, indicating that the early and late stages of fermentation were distinct. Based on PC2, the first-day fermentation sample was positioned on the lower side of the pot and the second- and third-day fermentation samples were positioned on the upper side, indicating that there were differences between days even within the early stage of fermentation.

The products of mixed-acid fermentation include mostly ethanol, acetic acid, lactic acid, succinic acid, and formic acid. If neutral fermentation occurs, 2,3-butanediol is produced from pyruvate through acetoin. 2,3-Butanediol is mostly produced by bacteria such as *Bacillus* and *Enterobacter* [24]. In this experiment, 2,3-butanediol was not detected.

Quantitative analyses of *makgeolli* metabolites during fermentation. Table 1 summarizes the major metabolites that were identified during fermentation of *makgeolli* by using UHPLC-Q-TOF MS in the positive and negative ion modes. Sixteen amino acids (including phenylalanine), the nonprotein amino acid GABA, and four organic acids (including citric acid) were identified. The quantitative analysis of the identified materials indicated that the contents tended to increase as the fermentation period increased (Table 2). It has been reported that amino acids are produced by the enzymatic action of microorganisms during fermentation of the protein contained in rice, the major raw material in *makgeolli* production, and that these

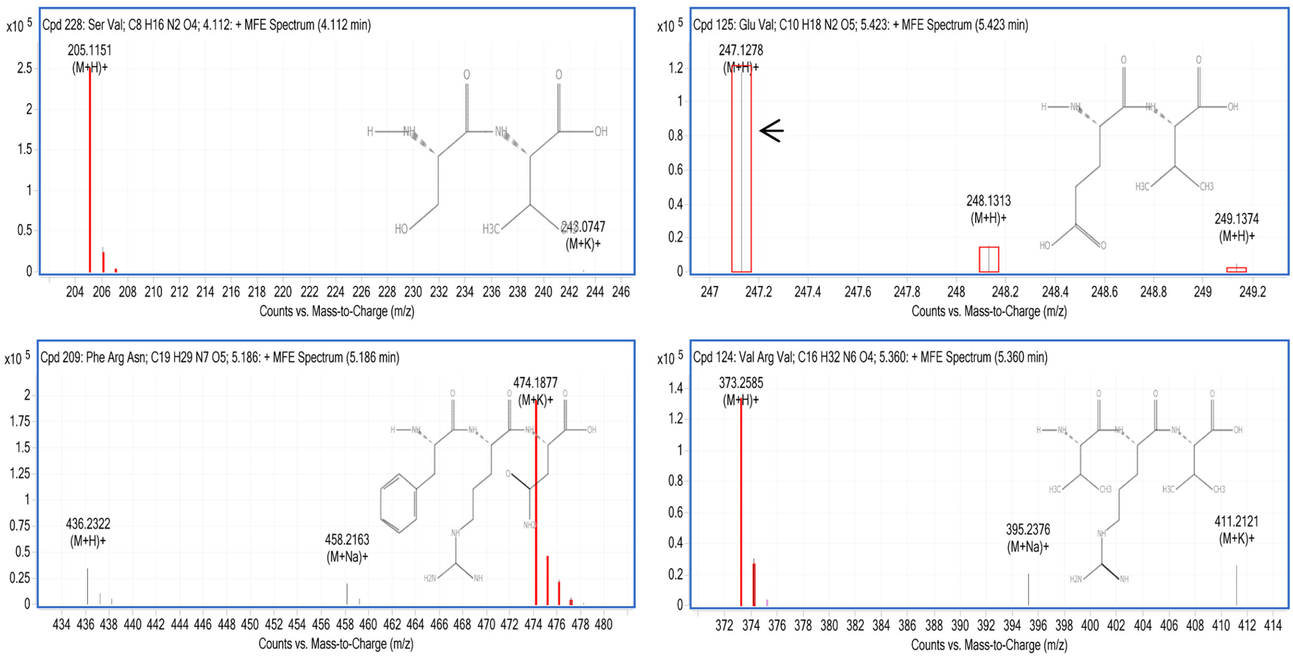

Fig. 4. Electron ionization mass spectra of $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ ions of Ser-Val, Glu-Val, Phe-Arg-Asn, and Val-Arg-Val.

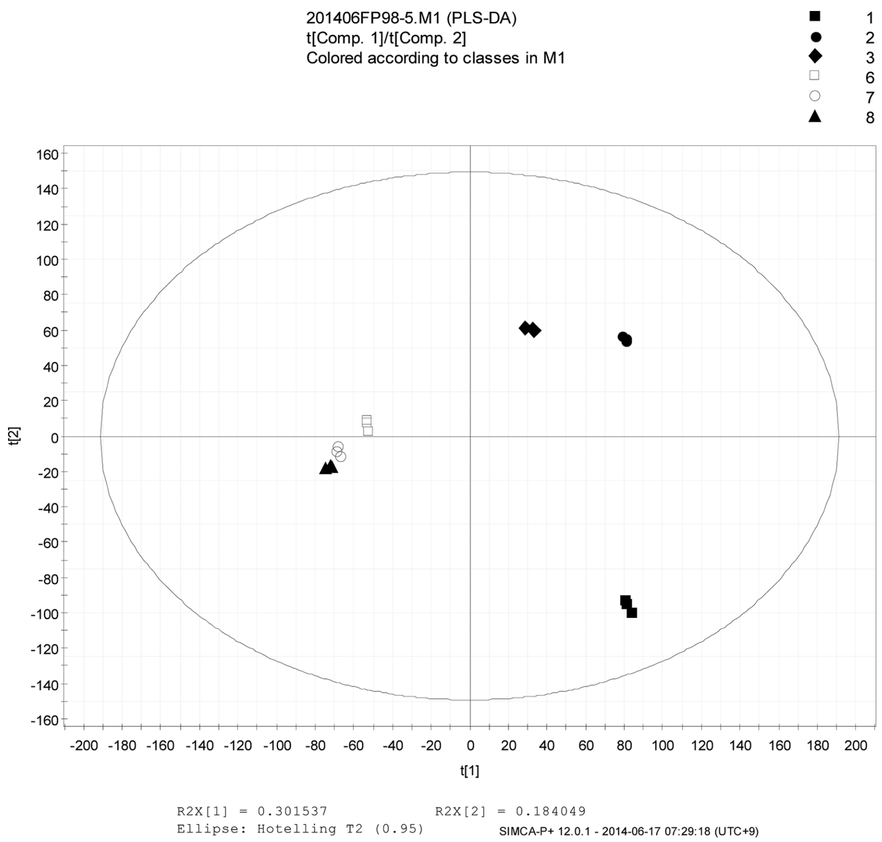

Fig. 5. Partial least squares-discriminant analysis score plot derived from ultra-high-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry profiles of *makgeolli* brewed with *Saccharomyces cerevisiae* Y98-5 during the fermentation period (■, 1; ●, 2; ◆, 3; □, 6; ○, 7; and ▲, 8 days). PC1 and PC2 account for 30.15% and 18.40% of the variance, respectively.

Table 1. Identification of major metabolites of Y98-5 *makgeolli* by UHPLC-Q-TOF MS in the positive and negative ion modes

No.	RT	Identity	Formular $[M+H]^+$	Exact mass	Actual mass	Mass error (ppm)	MS fragment (ESI)
1	4.323	Phenylalanine	C9H12NO2	166.0863	166.0867	2.408	120.08, 131.05
2	4.418	Tyrosine	C9H12NO3	182.0812	182.0825	7.139	136.07, 165.05
3	4.549	Leucine	C6H14NO2	132.1019	132.102	0.757	86.09
4	4.648	Isoleucine	C6H14NO2	132.1019	132.1017	−1.5140	86.09
5	4.692	Methionine	C5H12NO2S	150.0583	150.0583	0.00	104.05, 132.10
6	5.039	γ-Amino-n-butyric acid	C4H10NO2	104.0706	104.0710	3.844	86.06, 87.04
7	5.109	Valine	C5H12NO2	118.0863	118.0859	−3.387	72.08
8	5.303	Gluatamic acid	C5H10NO4	148.0604	148.061	4.052	84.04, 102.05, 130.05
9	5.415	Threonine	C4H10NO3	120.0655	120.0655	0.00	74.06, 102.05
10	5.55	Aspartic acid	C4H8NO4	134.0448	134.0448	0.00	88.03, 116.03
11	5.572	Serine	C3H8NO3	106.0499	106.0496	−2.829	60.04, 88.04
12	5.714	Alanine	C3H8NO2	90.055	90.0544	−6.663	44.049
13	5.955	Glycine	C2H6NO2	76.0393	76.0379	−18.412	48.05, 59.06
14	6.047	Proline	C5H10NO2	116.0706	116.0703	−2.585	70.06
15	8.457	Arginine	C6H13N2O4S2	175.119	175.1199	5.139	156.07
16	8.872	Histidine	C6H10N3O2	156.0768	156.0775	4.485	110.07
17	9.099	Lysine	C6H15N2O2	147.1128	147.1131	2.039	121.05, 130.08
			Formular $[M-H]^-$				
18	7.324	Malic acid	C4H5O5	133.0142	133.0139	−2.255	75.0, 87.0, 114.9
19	7.638	Lactic acid	C3H5O3	89.0244	89.0248	4.493	44.99, 87.00
20	9.730	Citric acid	C6H7O7	191.0197	191.0188	−4.712	68.99, 112.98
21	11.359	Succinic acid	C4H5O4	117.0193	117.0192	−0.855	68.99, 112.98

UHPLC-Q-TOF MS, ultra-high-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry; ESI, electrospray ionization.

Table 2. Quantitative analysis of major Y98-5 *makgeolli* metabolites during fermentation, using UHPLC-Q-TOF MS

	Makgeolli metabolite (μg/mL)					
	1 day	2 days	3 days	6 days	7 days	8 days
Phenylalanine	112.04 ± 0.36	167.64 ± 2.75	203.95 ± 1.56	289.63 ± 7.07	310.44 ± 7.75	323.83 ± 4.86
Tyrosine	120.93 ± 1.52	195.01 ± 4.08	243.14 ± 1.69	327.75 ± 11.00	344.31 ± 6.75	362.77 ± 7.48
Leucine	182.98 ± 3.00	184.03 ± 4.76	229.01 ± 3.35	392.45 ± 12.85	431.21 ± 4.21	463.13 ± 9.46
Isoleucine	49.73 ± 2.74	49.63 ± 1.81	55.10 ± 3.38	76.96 ± 1.95	81.89 ± 1.73	86.47 ± 1.48
Methionine	38.45 ± 0.34	40.63 ± 0.45	43.13 ± 0.42	75.55 ± 2.06	83.82 ± 1.70	90.85 ± 1.89
γ-Amino-n-butyric acid	12.36 ± 0.37	13.59 ± 0.88	11.41 ± 0.79	14.77 ± 0.73	15.45 ± 1.38	14.9 ± 0.27
Valine	45.43 ± 0.18	53.85 ± 0.20	60.72 ± 0.53	92.74 ± 1.28	103.69 ± 2.45	106.67 ± 1.77
Gluatamic acid	152.33 ± 3.46	184.80 ± 6.57	229.50 ± 3.89	282.43 ± 9.25	298.79 ± 5.94	309.30 ± 1.18
Threonine	40.02 ± 0.26	43.00 ± 0.78	44.48 ± 0.30	62.71 ± 1.50	66.70 ± 1.09	69.66 ± 1.99
Aspartic acid	58.62 ± 3.03	55.54 ± 1.23	67.56 ± 1.13	96.76 ± 4.22	105.43 ± 1.32	113.45 ± 3.93
Serine	45.01 ± 0.98	51.27 ± 6.97	54.69 ± 2.78	92.00 ± 3.28	92.03 ± 13.30	104.19 ± 4.08
Alanine	94.59 ± 1.04	107.16 ± 3.07	134.81 ± 2.15	118.32 ± 1.31	196.72 ± 2.03	193.39 ± 2.01
Glycine	72.22 ± 0.08	115.76 ± 0.68	122.01 ± 2.44	150.04 ± 5.38	152.08 ± 2.48	157.1 ± 1.37
Proline	67.82 ± 1.33	140.63 ± 3.89	179.22 ± 1.63	227.70 ± 4.70	242.98 ± 7.26	257.62 ± 4.53
Arginine			4.59 ± 0.16	6.10 ± 0.05	5.83 ± 0.77	9.76 ± 1.63
Histidine	90.58 ± 9.63	106.37 ± 10.44	97.67 ± 3.18	119.78 ± 3.72	126.96 ± 1.71	129.80 ± 11.41
Lysine	30.13 ± 7.77	51.89 ± 3.53	60.47 ± 5.01	99.86 ± 7.09	127.04 ± 2.84	128.79 ± 5.46
Malic acid	49.4 ± 1.2	93.3 ± 8.6	232.3 ± 6.0	494.8 ± 27.1	514.1 ± 24.1	529.06 ± 21.1
Lactic acid	74.4 ± 4.5	268.5 ± 6.3	535.9 ± 11.7	717.5 ± 45.4	722.7 ± 59.6	730.8 ± 41.1
Citric acid	4298 ± 12.2	5046 ± 120.5	5203 ± 40.3	5159 ± 76.1	5088 ± 54.8	5070 ± 66.3
Succinic acid	116.1 ± 4.3	360.4 ± 13.7	515.4 ± 6.6	628.9 ± 40.3	648.4 ± 32.1	679.3 ± 17.8

UHPLC-Q-TOF MS, ultra-high-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry.

constituents affect the taste of *makgeolli* [25]. It is known that *makgeolli* must contain free amino acids that produce a balance of sour, savory, sweet, and bitter tastes, and that higher amino acid contents are better [26]. At the early stage of Y98-5 *makgeolli* fermentation, the major amino acids were leucine, glutamic acid, tyrosine, and phenylalanine. Upon the completion of fermentation, the contents of alanine, proline, and glycine had increased, and these amino acids were identified as major amino acids of the *makgeolli*, along with the major amino acids identified at the early stage of fermentation. Although the major amino acids identified upon the completion of Y98-5 *makgeolli* fermentation included tyrosine and leucine, these amino acids were not among the major amino acids identified in *yakju* by Lee [27] (arginine, alanine, glutamic acid, serine, and glycine) or by Cheong *et al.* [28] (alanine, proline, phenylalanine, and glutamic acid). Tyrosine is the starting material for the production of neurotransmitters, including dopamine, and thus has been proposed as a treatment for depression. The high amount of leucine observed at the early stage of fermentation may be due to differences in the raw rice, *koji*, and yeast used in this study compared with previous studies. Leucine can be used to produce glucose when there is no intake of food and thus helps to maintain and regulate blood glucose levels. Among the amino acids, leucine had the highest content of 463.13 μg/mL on the eighth day of Y98-5 *makgeolli* fermentation, and the tyrosine content was 362.77 μg/mL, the second highest.

Glutamic acid aids sugar and fat metabolism and produces an umami taste [29]. The concentration of glutamic acid in the Y98-5 *makgeolli* on the eighth day of fermentation was 309.3 μg/mL. The concentration of alanine, which plays an important role in metabolism, aids liver detoxication, and produces a sweet taste, was 193.39 μg/mL. The concentration of phenylalanine was 232.83 μg/mL; this amino acid is effective for weight loss and lipid profile improvement because it elicits the secretion of cholecystokinin in the intestine, which reduces hunger by stimulating the satiety center of the brain. Valine, a 2-amino valeric acid, can be obtained from the hydrolysis of albumin and is synthesized through the amination of 2-oxoisovaleric acid by valine aminotransferase. It is a precursor of pantothenic acid and has properties similar to those of leucine. Thus, isolation of pure valine from protein hydrolysates is relatively difficult. Valine protects liver function and has a slightly bitter taste in addition to a sweet taste [30]. On the eighth day of fermentation, the concentration of valine was 106.67 μg/mL. The concentration of methionine, an amino acid that plays an important role as a precursor of *S*-adenosylmethionine and promotes fat metabolism, was 90.85 μg/mL. A low amount of GABA, a primary inhibitory neurotransmitter in the brain [31], was observed during *makgeolli* fermentation. The highest amount, 15.45 μg/mL, was observed on the seventh day of fermentation.

Organic acids are major constituents that contribute to the taste of fermented alcoholic beverages such as sake and wine [32, 33]. The major organic acid of Y98-5 *makgeolli* was citric acid. The maximum amount of citric acid, which has a fresh sour taste, was 5.088 mg/mL on the seventh day of fermentation. Lactic, succinic, and malic acids

(529~730 μg/mL) were the next most abundant organic acids, in that order; the concentrations of all three tended to increase until the eighth day of fermentation.

In conclusion, in *makgeolli* brewed with *S. cerevisiae* Y98-5 (isolated from traditional Korean *nuruk*) and *koji*, the amino acid, GABA, and organic acid contents increased during the fermentation period, and the citric acid content reached a maximum on the seventh day of fermentation. The amounts of tyrosine, which is involved in stimulating and invigorating the brain, and leucine, which functions in blood sugar regulation, were high. For the metabolomic study of traditional alcoholic beverages, more metabolite libraries are needed. Although wine yeasts and baker's yeasts are currently imported from foreign countries and used for the brewing of *makgeolli*, the above results demonstrate the nutritional superiority of a domestic yeast isolated from traditional Korean *nuruk*. The results of this study could form the basis for the invigoration of domestic yeast.

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