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 strains [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-
converting-enzyme inhibitor [19] and an antidepressant β-
secreasase 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 Goeji 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 ultrahigh-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 (N₂) 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   | –1.514           | 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| Glutamic acid           | C5H10N5O4        | 148.0604   | 148.0601   | 4.052            | 84.04, 102.05, 130.05 |
| 9   | 5.415| Threonine               | C6H15N2O2        | 120.0655   | 120.0655   | 0.00             | 74.06, 102.05     |
| 10  | 5.55 | Aspartic acid           | C4H3NO4          | 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    | –0.663           | 44.049            |
| 13  | 5.955| Glycine                 | C2H6N2O2         | 76.0393    | 76.0379    | –18.412          | 48.05, 59.06      |
| 14  | 6.047| Proline                 | C5H10N2O2        | 116.0706   | 116.0703   | –2.585           | 70.06             |
| 15  | 8.457| Arginine                | C6H14N2O2S       | 175.119    | 175.1199   | 5.139            | 156.07            |
| 16  | 8.872| Histidine               | C6H13N2O2        | 156.0768   | 156.0775   | 4.485            | 110.07            |
| 17  | 9.099| Lysine                  | C6H15N2O2        | 147.1128   | 147.1127   | 2.039            | 121.05, 130.08    |
| 18  | 7.324| Malic acid              | C4H5O4           | 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.
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 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

| 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   | 57.55 ± 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  |
| Glutamic acid     | 152.33 ± 3.46 | 184.80 ± 6.57  | 229.50 ± 3.89  | 282.43 ± 9.25  | 298.79 ± 5.94  | 309.3 ± 7.48   |
| Glutamic acid     | 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  | 189.08 ± 3.64  | 196.72 ± 2.03  | 193.49 ± 3.01  |
| Glycine           | 72.22 ± 0.08  | 115.76 ± 0.68  | 122.01 ± 2.44  | 150.04 ± 5.38  | 152.08 ± 2.48  | 157.4 ± 1.37   |
| Proline           | 67.82 ± 1.33  | 140.63 ± 3.89  | 179.22 ± 1.63  | 227.40 ± 7.40  | 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    | 129.80 ± 11.41 | 129.80 ± 11.41 |
| Histidine         | 90.58 ± 9.63  | 106.37 ± 10.44 | 97.67 ± 3.18   | 119.78 ± 3.72  | 126.96 ± 1.71  | 126.96 ± 1.71  |
| 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       | 429.8 ± 12.2  | 504.6 ± 120.5  | 520.3 ± 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.
(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, 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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