Microbial Diversity and Volatile Flavor Changes during Gayangju Fermentation, a Traditional Korean House Rice Wine
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Abstract: Physicochemical changes in fermented alcoholic beverages are significantly related to microbial community development during fermentation. Due to its unusually long fermentation, Gayangju, a traditional Korean house rice wine fermented with nuruk as the traditional starter, gives rise to a strong yeast community and, therefore, a high ethanol concentration and different flavors. However, no detailed analysis has been examined. Changes in microbial community structure during Gayangju fermentation were examined using both culture-dependent and culture-independent methods. During fermentation, Saccharomyces cerevisiae and Saccharomycopsis fibuligera were dominant during all stages of the fermentation. In contrast, Candida parapsilosis, Hanseniaspora guilliermondii, Pichia anomala, Malassezia cuniculi and P. fermentans were identified as minor. P. anomala appeared after the second brewing and then remained constant. Among the 19 compounds identified in this study as order-active compounds, 2-methyl-1-butanol (isoamyl alcohol) was the major compound that increased during the long fermentation stage. Most of the odor-active compounds such as 2,3-butanediol, 3-methyl-1-butanol, ethyl tetradecanoate, ethyl decanoate, ethyl dodecanoate, butanoic acid, 3-methylbutanoic acid (isovaleric acid), 2-methylbutanoic acid, 2-methyl-1-propanol, ethyl acetate, ethyl caprylate, 2-phenylethanol, and 3-methylbutyl acetate increased as the fermentation progressed during 68 days of fermentation, which showed significant differences in the concentrations of odor-active compounds of commercially fermented makgeolli.

Keywords: microbial diversity; volatile flavor; Korean traditional house rice wine; Gayangju

1. Introduction

Gayangju is a Korean rice wine (makgeolli) traditionally home brewed since ancient times, and it has been widely consumed in Korea because of its deep taste and desirable flavor [1]. Compared to the typical commercial procedure for brewing Korean rice wine, which involves very short fermentation steps without nuruk as the fermentation starter, the Gayangju fermentation procedure is different regarding its unusually long fermentation steps, as shown in Figure 1 [2]. Due to its unusually long fermentation steps and aging processes—lasting two months with the addition of nuruk compared to commercial fermented rice wine, which takes only approximately five days without nuruk—Gayangju generally exhibits extraordinarily high ethanol concentrations of up to 18–20% (w/w) and chemical diversity including alcohols, esters, organic acids, fatty acids, and amino acids, which might lead to distinguished flavors and quality.

Gayangju is generally produced through three main processes: (1) alcohol fermentation with a natural starter culture (nuruk) at 25 °C for three days; (2) alcohol fermentation with the addition of newly prepared raw materials (mainly cooked rice) for two additional days; (3) post-process with aging without sterilization. Compared to commercial processes, this fermentation process largely affects not only the ethanol content but also the overall quality, including flavors. Nuruk is prepared by the natural solid fermentation of moistened ground rice or wheat sources, allowing for the complex mixed growth of molds (Aspergillus, Rhizopus, and Mucor spp.), yeasts (Saccharomyces, Pichia, Candida, Torulopsis, and Hansenula...
spp.), and lactic acid bacteria (*Leuconostoc*, *Pediococcus*, and *Lactobacillus* spp.) [3–5]. All these microorganisms can affect the physiological and biochemical properties of the final fermented products through metabolic pathways of alcohol and lactic acid fermentation after saccharification by molds that release amylase and glucoamylase [5]. Finally, the post-processing aging stage promotes the esterification between acids and alcohols, improving the flavor profile. Various volatile components developed via the alcoholic and lactic acid fermentation stage can be converted into small molecules, and yeasts can be used as basic materials for complex, volatile profiles affecting the physicochemical quality of *makgeolli* [5,6]. During the long period of Gayangju fermentation, in particular, unique microbial consortiums might result in different microorganism developments. Although many studies have shown microbial diversities in *makgeolli* or nuruk, there has been no information on the microbial diversity or biochemical properties of Gayangju during its fermentation process.

![Flow sheet depicting the preparation of fermented traditional Korean rice wine, Gayangju.](image)

A few volatile compounds in commercially available *makgeolli* have been identified, consisting of 45 major volatile compounds composed of 33 esters, 8 alcohols, 1 aldehyde, 1 acid, 1 phenol, and 1 terpene. sp. [6,7]. The key aroma-active components are the esters such as ethyl decanoate, ethyl (Z)-octadec-9-enoate, ethyl octanoate, 2-phenethyl acetate, ethyl acetate, 3-methylbut-1-yl ethanoate, ethyl hexadecanoate, ethyl 9,12-octadecadienoate, ethyl dodecenoate, and ethyl tetradecanoate, usually known as by-products of yeasts [6,7]. The development of volatile compounds in *makgeolli* is influenced by several factors related to the fermentation process, including starting materials, processing techniques, and specific microorganisms. However, information regarding the volatile flavor compounds present in Gayangju and its change during fermentation is still lacking.

Thus, this study aimed to determine the microbial community succession and flavor change profiles during Korean traditional house rice wine Gayangju fermentation by adopting both culture-dependent and culture-independent methods. For the microbial community change analysis, a denaturing gradient gel electrophoresis (DGGE) method was used, and a gas chromatography-mass spectrometry (GC-MS) instrument with an
automatic purge and trap concentrator was used for one-step direct flavor change analysis with low detection limits [8].

2. Materials and Methods

2.1. Gayangju Fermentation

Gayangju fermentation was carried out at the JeonJu Korean Traditional Wine Museum (http://urisul.net/ accessed on 6 June 2021). Rice (non-glutinous and glutinous rice) was obtained from Buan, Korea, and nuruk was acquired from Songhak Gokja (Gwangju, Korea), the representative manufacturing company for fermented nuruk production in Korea. Non-glutinous rice (1 kg) was washed and soaked briefly in distilled water at room temperature overnight and then ground. The rice cake made with boiled water was mixed with water (7 L) and nuruk (1.0 kg, 10% w/w of the total grain source). The mixture was placed in a crock and initially fermented at 25 °C for approximately 36 h until the temperature of the fermenting material reached 37 °C. After cooling to 10 °C for 36 h, the mixture was used as the starter material (first-stage mash), while glutinous rice (Oryza sativa var. glutinosa 8 kg) was washed, soaked overnight, and then steamed. The first-stage mash was further mixed with the heated glutinous rice, and the mixture was used as the second-stage mash for alcoholic fermentation. The second fermentation was conducted at 25 °C for 48 h. The final fermentation and aging stage was carried out at 15 °C for 35 days. Finally, the obtained Gayangju was filtered through filter cloth and bottled as the final product (Figure 1). The samples were collected every 6 h during the first fermentation, every 12 h during the second fermentation, and every week during the rest of the nine weeks (until the end of the 5-week fermentation and 4-week storing periods) to be analyzed during the index period. Sampling was done in triplicate from different jar fermentors. As a control, commercial rice wine was prepared as described below. A total of 200 g of nonglutinous rice was rinsed and then soaked in tap water for 3 h. After draining, the rice was immediately steamed for 40 min and quickly cooled by being spread out thinly on an aluminum pan. Commercial rice wine fermentation was carried out in a 1.5 L glass bottle along with the distilled water (300 mL), glucoamylase (800 GAU), α-amylase (1350 BAU), and Saccharomyce cerevisiae (equivalent to 10% of the total volume) at 25 °C.

2.2. Physicochemical Analysis

During the fermentation, measurements of pH, total acidity, total soluble solids, residual sugar, and ethanol were performed. pH was measured using a pH meter (Orion model 710; Thermo, Beverly, MA, USA), and total acidity was measured with a 0.1 N NaOH solution. Total soluble solids were measured with a refractometer (PAL-α, 0–85 Brix; Atago, Tokyo, Japan), and the residual sugar content was analyzed using the DNS method (Miller, 1959). For the analysis of alcohol content, a GC system (HP 6890 series; Agilent Technologies, Waldbronn, Germany) and a J&W DB-5 capillary column (30 m × 0.25 mm id, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) were used, with He as the carrier gas [9]. Isopropanol was used as the internal standard for the quantitation.

2.3. Free Amino Acids Analysis

For the free amino acid analysis, ethanol extraction was conducted with 70% (v/v) ethanol using a Branson 2510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA) at 80 °C for 15 min. After eliminating the lipid phase by adding 20 mL of ether, the obtained solution was dried by evaporating under reduced pressure at 40 °C using a rotary evaporator (EYELA; Tokyo Rikakikai Co., Tokyo, Japan). The sample re-dissolved in sodium citrate buffer (pH 2.2) was analyzed with an amino acid analyzer with an LCA60/Na cation separation column (150 × 4.6 mm). The column temperature was increased from 50 to 80 °C. Sodium citrate buffers (pH 3.3, 4.3, 5.2, and 10.1) and ninhydrin solution were used as mobile phases at flow rates of 50 and 25 mL/h, respectively.
2.4. Microbiological Analysis

For the counts of yeast, total aerobic mesophilic bacteria, and lactic acid bacteria (LAB), 100 µL of a decimal dilution in 0.85% sterile saline solution was spread onto YM agar plates supplemented with penicillin (20 units/mL), streptomycin (40 µg/mL, Sigma-Aldrich, St. Louis, MO, USA), nutrient agar (Merck, Darmstadt, Germany), and MRS agar (Difco, Franklin Lakes, NJ, USA) plates with cycloheximide (Sigma), respectively. Incubation was conducted at 29 °C for 48 h for yeast and at 37 °C for 30 h for total aerobic mesophilic bacteria and LAB. The number of viable cells was determined by counting in triplicate, and the results were expressed as log cfu/mL. For the PCR-DGGE analysis as a culture-independent method, DNA was extracted using a NucleoSpin® Food genomic DNA extraction kit (Macherey-Nagel, Duren, Germany), following the manufacturer’s protocol for the purification of total genomic DNA. For the analysis of fungal diversity, fragments of the fungal gene at the 26S rRNA D1/D2 region were generated using the eukaryotic universal primer NL1 containing a CG-clamp and LS2 [9]. For the analysis of bacterial diversity, the V3 region of 16S rDNA was amplified by PCR using the universal bacterial primer 357F containing a CG clamp and 517R. DGGE analysis was performed in 8% (w/v) polyacrylamide gels (acylamide: bisacrylamide 37.5:1) using a Dcode apparatus (BioRad, Richmond, CA, USA). A denaturing gradient gel from 30% to 60% was run at 125 V and 60 °C for 5.5 h and stained with an EtBr solution (0.5 µg/mL). Bands visualized under UV light (ChemiDoc XRS Imaging System, Bio-Rad) were re-amplified using the same primers without the GC clamp. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and then analyzed with an automated DNA sequencer (ABI PRISM 3700; Applied Biosystems, Foster City, CA, USA). The identity of the sequences was determined by the BLASTN algorithm in the GenBank database.

2.5. Volatile Flavor Analysis

Volatile flavor compounds were analyzed using a GC/MS QP 2010 plus (Shimadzu, Kyoto, Japan) with an automated Purge & Trap Sampler JTD-505III (Japan Analytical Industry, Tokyo, Japan). In addition, GC-MS analysis for volatile flavor compounds was performed as described by Song et al., with 2-Methyl-3-heptanone used as an internal standard [10].

2.6. Statistical Analysis

All analyses were performed in triplicate. The data were analyzed using the SPSS ver. 16.0 program (SPSS Inc., Chicago, IL, USA). Statistical evaluation was performed using the one-way ANOVA, and significant differences were determined using Duncan’s multiple range tests at \( p < 0.05 \). The correlation between variables was determined by Pearson’s correlation analysis.

3. Results and Discussion

3.1. Physicochemical Change during Gayangju Fermentation

During the traditional Korean makgeolli process by the second brewing (fermentation for 42 days and storage for 14 days), the total acidity, pH, and alcohol and sugar contents were investigated. Korean commercial rice wines exhibited an acidic pH of 3.5–4.5 and a total acidity of 0.35–0.70 [8]. As the key factors related to fermentation properties, the overall pH and acidity could be changed by the production of various organic acids by the microbes—mainly, LAB strains. In this study, the total acidity rapidly increased to 0.69% during the first brewing process of three days, and then, after adding a second rice source, the acidity (0.24%) increased to 0.55% during the second brewing and aging periods (Figure 2A). Likewise, the initial pH of 6.52 sharply decreased to 3.68, and the pH value remained at 4.07–4.35 for the rest of the fermentation process. During the first brewing process, glutinous rice was saccharified, especially on the first day, and the reducing sugar content increased from the initial 3.7% to 4.2% on day 1 and then decreased to 2.6%, while the alcohol content rapidly increased to 17.4% (Figure 2B). Thereafter, the sugar content
was again increased to 5.5% by adding a second rice source; however, this decreased to 4.3% on day 6. In contrast, the alcohol content (6.10%) on day 3 again increased to 16.0% during the second brewing process, and then the aging periods yielded a further increase to 19.2% on day 40. In addition, refrigeration storage for 12 days resulted in a further increase in acidity to 0.73%, and on day 70, the alcohol content decreased to 18.7%. Additionally, the sugar content rose slightly to 5.2%. In this study, the composition and contents of the free amino acids in the samples were also analyzed, and the results are shown in Figure 3. A significant difference in the total free amino acid contents between Gayangju (26.4 mg/mL) and commercial makgeolli (11.12 mg/mL) was observed. The contents of glutamic acid and aspartic acid (associated with flavors of ‘richness’), as well as threonine and alanine (associated with the taste of ‘sweetness’), were higher in Gayangju than they were in commercial makgeolli. In addition, the arginine and leucine (associated with flavors of ‘bitterness’) were significantly higher than commercial makgeolli (p < 0.01). Notably, Gayangju showed about two-times-greater contents of aspartic acid, which is the main influence on savory flavors compared to commercial makgeolli. Additionally, threonine, serine, and alanine, which are the main influences on sweetness, were 1.5- to 2-fold higher in the samples of Gayangju. The levels of tryptophan were significantly higher in Gayangju, which is known to occur only in fermentation and may be a potential precursor of an aroma compound, 2-aminoacetoephone (AAP). Although an increase in AAP is significantly related to the ‘untypical aging off-flavor’ (UTA) during fermentation, it seems that the high-level accumulation of tryptophan indicates no AAP progress [11].

Figure 2. Changes in physicochemical characteristics during traditional Korean rice wine (Gayangju) fermentation. (A) pH (○) and total acidity (●); (B) contents of alcohol (○), reducing sugars (●), and total soluble solids (●).
3.2. Microbial Change during Makgeolli Fermentation

The populations of yeast, LAB, and total aerobic mesophilic bacteria during the fermentation and storage of makgeolli were estimated by the plating method (Figure 4). The LAB population numbers were similar to the total aerobic mesophilic bacteria during the overall fermentation and storage periods. Initially, the LAB count was 7.59 log cfu/mL; after 24 h of fermentation, the population reached 9.34 log cfu/mL and remained at similar levels until day 14. Through the continuous aging process, the LAB population began to decline, and a LAB count of 7.18 log cfu/mL was detected on day 42. However, the further storage periods yielded an increase to 7.53 log cfu/mL on day 70. At the beginning of the fermentation, the yeast population was approximately 7.70 log cfu/mL and increased over the following days, reaching the maximum population during the second brewing (9.09 log cfu/mL on day 4). After this time, the yeast population remained for two days and then decreased steadily until further aging and storage periods. On the 70th day, the yeast population was 6.16 log cfu/mL.

Figure 3. Free amino acids analysis of Gayangju and the commercial rice wine makgeolli.

Figure 4. Changes in microbial counts (yeast, total bacteria, and lactic acid bacteria) during traditional Korean rice wine fermentation.
By a culture-independent method, the species composition and the dynamics of the fungal and bacterial community during the different fermentation and storage times were investigated (Figure 5), and a wide diversity of bacterial species existed in all the processes. Of the 17 bands analyzed, the DNA sequences of ten bands corresponded to LAB, revealing that it was the major bacterial group in makgeolli fermentation. Mainly, Lactobacillus curvatus (band 3), Lactobacillus sakei (band 4), and Pantoea agglomerans (band 5) were found to be predominant during the entire process, including the storage period. The species appeared as inferior bands in nuruk at day 0 but as dominant bands after one day. On the other hand, Lactiplantibacillus plantarum (band 1) was detected after one day and remained as a band with weak intensity over the following days. P. pentosaceus (band 8) was consistently detected as one of the marginal species throughout the overall fermentation process. In addition, the bands corresponding to L. sakei (band 2), L. sakei (band 15), Lacticaseibacillus casei (band 16), and Lacticaseibacillus paracasei (band 17) also persisted throughout the fermentation process. As other bacteria genera, Enterococcus faecium (band 9), Enterobacter sp. (bands 11, 13), and P. inopinatus (band 12) were identified in the nuruk sample but disappeared as the fermentation progressed.

Generally, the fungal communities during wine fermentation were far simpler than the bacterial communities (4). To date, several species of Aspergillus, Rhizopus, Candida, Saccharomyces, Wickerhamomyces, and Saccharomycopsis sp. have been found in the spontaneous fermentation of Korean rice wines, of which S. cerevisiae was the dominant yeast throughout the entire process, representing an average of above 90% [3,12]. Meanwhile, the fungal community of the nuruk (the starter) was more complex, indicating the diverse mycolonal dynamics of Aspergillus, Cladosporium, Eurotium, Lichtheimia, Mucor, Penicillium, and Rhizopus sp., among others; however, they were distributed less than 0.1% [4,13]. Unlike rice wine, Pichia sp. was the most dominant yeast, whereas a representative alcohol fermentation strain, S. cerevisiae, was detected in only some of the nuruk samples [13]. In this study, fungal communities during the makgeolli fermentation were also assessed, and PCR-DGGE based on the analysis of 26S DNA clone libraries resulted in a total of 12 bands (Figure 5B). Yeast species including Saccharomyces cerevisiae (bands 1, 5, 10), Candida parapsilosis (band 2), Hanseniaspora guilliermondii (band 3), Saccharomycopsis fibuligera (bands 7, 8), Pichia anomala (band 4), Malassezia cuniculi (band 6), and Pichia fermentans (band 9) were identified, in which S. cerevisiae and S. fibuligera were found to be dominant, observed in all stages of the fermentation. Specifically, S. cerevisiae (band 1) also appeared as the dominant band in the nuruk material, and as other bands corresponded to S. cerevisiae, band 5 disappeared after entering the aging stage, while band 10 appeared during the first brewing process. In the case of S. fibuligera, band 8 was observed in the nuruk at all stages of the makgeolli process, while band 7 appeared during the first brewing process but remained as a superior band. In addition, P. anomala (band 4) appeared after the start of the second brewing and then remained constant; however, its band intensity was weak. With respect to the other strains, C. parapsilosis (band 2), H. guilliermondii (band 3), M. cuniculi (band 6), and P. fermentans (band 9) were detected as rare, minor strains. As mold sources, bands corresponding to Rhizopus sp. (band 11) and Aspergillus oryzae (band 12) were also identified in the nuruk material but disappeared during the first brewing. Most of the detected fungi in this research were reported in many kinds of fermented rice liquors. Meanwhile, no significant variation was observed in their community after the start of the storage. In rice wine fermentation, saccharification in the early stages is mainly due to molds from nuruk [5,12]. However, these molds are numerically inferior to yeast and constitute below 5% of the total microbe, and then the molds rapidly decrease during the fermentation process [5,12]. Meanwhile, S. fibuligera commonly exists in amylolytic yeast in Indigenous food fermentation using starchy substrates such as rice and cassava [14]. Although it is also thought of as a foodborne and dimorphous yeast, S. fibuligera has received increasing attention, as it can secrete amylase, glucoamylase, β-glucosidase activity, and trehalose [14]. Jung et al. (2012) reported that S. fibuligera existed at high levels during the increase in glucose concentration and was replaced by S. cerevisiae as the ethanol concentration in-
S. fibuligera is also used as the main amylase producer for ethanol production from starch [14]. P. anomala has a positive role in food preservation and is well known as a flavor-enhancing (especially ester-producing) yeast in food and beverage fermentation [12]. In recent years, the significance of non-Saccharomyces species in winemaking has attracted the interest of winemaking researchers, and they contribute to the final taste and flavor of wines. Some non-Saccharomyces yeasts, such as Candida sp., can negatively affect the aroma and flavor of wine [15].

3.3. Volatile Compounds Change in Gayangju

Flavor and aroma are essential distinguishing characteristics of fermented wine and result from raw materials, winemaking practices, yeast strains, and aging conditions [16]. Moreover, most of the volatile compounds responsible for the organoleptic characteristics in rice wine are directly or indirectly transformed from the constituent of rice metabolism [17]. The increment values of major order-active flavor components during Gayangju fermentation are shown in Table 1. Among the 19 compounds identified in this study as order-active compounds, 2-methyl-1-butanol (isoamyl alcohol) was the major compound that significantly increased during the long fermentation stage. Most of the odor-active compounds such as 2,3-butanediol, 3-methyl-1-butanol, ethyl tetradecanoate, ethyl decanoate, ethyl dodecanoate, butanoic acid, 3-methylbutanoic acid (isovaleric acid), 2-methylbutanoic acid, 2-methyl-1-propanol, ethyl acetate, ethyl caprylate, 2-phenylethanol, and 3-methylbutyl acetate increased as the fermentation progressed during the 68 days of fermentation, showing significant differences in the concentrations of odor-active compounds of commercially fermented makgeolli. PCA and HCA analyses were performed to understand the volatile flavor development change during the long fermentation stage of Gayangju. As shown in Figure 6, an overall PCA biplot was constructed with a total variance of 80.34%. PC1 was strongly and positively related to most volatile compounds occurring in the late stage of Gayangju fermentation, while it was strongly negatively related to the early stage of Gayangju fermentation. In addition, most of the volatile compounds are positively related to Gayangju and not commercially produced rice wine. Based on these properties, the Gayangju fermentation at different stages was differentiated with GY3, GY5, GY40, and GY68 positioned fully in the strongly positive PC1 region. The later aging stage samples were positioned in the positive PC1 region due to the elevated concentrations of most order-active compounds. During the spontaneous fermentation of wine, yeast produces higher alcohols and esters, and the compounds strongly influence the sensory properties of the resulting wine [16]. In this study, alcohols and esters also comprised the largest groups of compounds in rice wines. Most identified volatile compounds were common in many other fermented alcoholic beverages such as wine, beer, and rice wine [18,19]. Compounds including 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-methylbutyaldehyde, ethyl acetate, and 3-methylbutyl acetate were present in all rice wine samples. 2-methyl-1-butanol (fermented and malt-like order notes) as amyl alcohols usually converted from leucine through deamination and decarboxylation reactions during the long-term aging fermentation of wine, beer, and sake [20,21]. The significantly increased concentrations of 2-methyl-1-butanol in the long-term aging period of Gayangju could be related to the qualities of alcoholic beverages due to their characteristic fermented, malt-like, and alcoholic-like odor notes [20]. The commercial rice wine prepared by S. cerevisiae strain (Lalvin EC-1118) as an ethanol producer resulted in less small, diverse compounds of eight, with the lowest concentration of total compounds. Mainly, 2-methyl-1-propanol (isobutyl alcohol; sweet; fusel, spirituous, sweet pear/nutty) was only detected as a major compound in commercial rice wine, in which fusel alcohols are formed from branched-chain amino acids by yeast during alcohol fermentation [17]. S. cerevisiae is an essential fermentative microorganism that produces ethanol from glucose, fructose, and sucrose. It synthesizes both nutritive (amino acids and vitamins) and flavor-volatile compounds, such as ethyl esters (ethyl decanoate, ethyl dodecanoate, and ethyl tetradecanoate) that influence the quality and aromatic profile of beverages [12].
Figure 5. DGGE fingerprints of bacterial 16S rDNA (A) and eukaryotic 26S rDNA (B) during Korean traditional rice wine fermentation. The closest relatives of the fragments sequenced compared to the sequences retrieved from the GenBank database are as follows: (A1) Lactiplantibacillus plantarum (AY590777), (A2) Lactilactobacillus sakei (JN851763), (A3) Lactilactobacillus curvatus (JQ247525), (A4) Liquorilactobacillus satsumensis (AB362684), (A5) Pediococcus acidilactici (AB627837), (A6) Erwinia sp. (KC853200), (A7) Pantoea sp. (DQ122375), (A8) Pediococcus pentosaceus (AB236655), (A9) Pantoea agglomerans (DQ122373), (A10) Pantoea sp. (JF946788), (A11) Enterobacter cowanii (JQ660056), (A12) Pediococcus inopinatus (JN863658), (A13) Enterobacter cloacae (KF481919), (A14) Pantoea sp. (DQ122350), (A15) Liquorilactobacillus satsumensis (AB362684), (A16) Lacticaseibacillus casei, (A17) Lacticaseibacillus paracasei; (B1) Saccharomyces cerevisiae, (B2) Candida parapsilosis, (B3) Hanseniaspora guilliermondii, (B4) Pichia anomala, (B5) Saccharomyces cerevisiae (GU080046), (B6) Malassezia cuniculi (GU733708), (B7) Saccharomyces fibuligera (JX141337), (B8) Saccharomyces fibuligera (HM107786),
(B9) *Pichia fermentans* (JQ665247), (B10) *Saccharomyces cerevisiae* (JX141338), (B11) *Rhizopus* sp., (B12) *Aspergillus oryzae*. The similarity of all band sequences was ≥ 97% compared with those available in the GenBank database.

### Table 1. Changes in volatile flavor compounds in Gayangju fermentation.

| No. | Flavor Compounds          | Commercial Makgeolli | Gayangju Makgeolli |
|-----|---------------------------|----------------------|--------------------|
|     |                           | 3 Days | 5 Days | 15 Days | 40 Days | 68 Days |
| F1  | 2,3-Butanediol            | 0      | 1      | 2.15b   | 5.79c   | 5.43c   | 9.41d   |
| F2  | 3-Methyl-1-butanol        | 2.97a  | 1      | 2.09c   | 4.59d   | 3.56e   | 7.29f   |
| F3  | 2-Methyl-1-butanol        | 0      | 1      | 2a      | 6.79b   | 59.35c  | 691.53d |
| F4  | 2-Methyl-1-propanol       | 5.79   | 0      | 0       | 0       | 0       | 0       |
| F5  | Ethyl tetradecanoate      | 0      | 1      | 1.59b   | 3.02c   | 4.53d   | 4.92d   |
| F6  | Ethyl dodecanoate         | 0      | 1      | 2.20b   | 5.26c   | 5.15c   | 5.54d   |
| F7  | Ethyl decanoate           | 0      | 1      | 2.36b   | 5.42c   | 4.45d   | 8.65e   |
| F8  | Methylbutanoic acid       | 0      | 1      | 3b      | 16.2c   | 4.05b   | 9.11d   |
| F9  | 2-Methylbutanoic acid     | 0      | 1      | 2.74    | 5.71    | 4.74    | 8.13    |
| F10 | 3-Methylbutanoic acid     | 0      | 1      | 2.67    | 1.9     | 5.37    | 8.63    |
| F11 | 2-Methylbutyraldehyde     | 0.85a  | 1      | 1.2     | 1.25    | 3       | 2.75    |
| F12 | 3-Methylbutyraldehyde     | 1.60a  | 1      | 1.67a   | 1.87a   | 0.23b   | 2.04a   |
| F13 | 2-Methylthio-1-propanol   | 0      | 1      | 2.51b   | 5.45c   | 4.89c   | 6.59d   |
| F14 | Ethyl acetate             | 0.69a  | 1      | 1.2a    | 1.7b    | 2b      | 2.2b    |
| F15 | Ethyl caprylate           | 0.1b   | 1      | 1.96c   | 2.38d   | 4.29e   | 6.19f   |
| F16 | 2-phenylethylthanol       | 0      | 1      | 2.23b   | 5.11d   | 4.32c   | 8.49e   |
| F17 | Ethyl pentadecanoate      | 0      | 1      | 1.22a   | 2.81b   | 2.69b   | 3.12c   |
| F18 | 3-Methylbutyl acetate     | 0.13a  | 1      | 1.74b   | 2.74c   | 4.07d   | 8.15e   |
| F19 | 2-Ethyl-1-hexene          | 0.07a  | 1      | 0.8a    | 6.07b   | 8.11c   | 9.43d   |

The increment values are calculated by the number of volatile flavor compounds in other samples divided by the number of volatile flavor compounds in a 3-day fermented Gayangju. Different small letters (a–f) indicate significant differences of values between commercial Makgeolli samples and Gayangju Makgeolli with different fermentation time (p < 0.05).

![Figure 6](image-url)

**Figure 6.** Biplot of the principal component analysis of volatile compounds from Korean traditional fermented *makgeolli*, *Gayangju*. pH, TA, RS, and EtOH indicate final pH, total acidity, soluble sugar, reducing sugar, and ethanol, respectively (C); The codes for flavor compounds (●) are defined in Table 1; As fermented *Gayangju* (○, GY), GY3, GY5, GY15, GY40, and GY68, indicate samples of different fermentation time of *Gayangju*. CO (△) indicates commercial Makgeolli.
4. Conclusions

This study was the first report to reveal the dynamics of microbial succession and the changes in flavor compounds during the long fermentation of Korean house rice wine, Gayangju. During fermentation, Gayangju maintains a strong yeast community, with a high ethanol concentration of 18.7%. Saccharomyces cerevisiae and Saccharomycopsis fibuligera were dominant at all stages of the fermentation, even in high ethanol circumstances. However, the non-saccharomyces of Candida parapsilosis, Hanseniaspora guilliermondii, Pichia anomala, Malassezia curvata, and P. fermentans were minor. The major bacterial groups in the Gayangju fermentation were Lactilactobacillus curvatus, Liquorilactobacillus satsumensis, and Pediococcus acidilactici during the entire process, including the storage period. The volatile compounds analyzed by a GC/MS with an automated Purge & Trap Sampler were made of 19 compounds, including 2-methyl-1-butanol (isoamyl alcohol) as the major compound that increased during the long fermentation stage. The significantly increased concentrations of 2-methyl-1-butanol in the long-term aging period of Gayangju could be related to the qualities of Gayangju with malt-like and alcoholic-like odor notes. Further studies regarding the relationships between the flavor profiles and the specific microbes can bring us closer to improving the quality of Gayangju and the efficiency of its production.

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References
1. Bae, S.M. *The Technology of Korean Traditional Liquor Making*; WooKok Publishing Company: Seoul, Korea, 2002.
2. Kim, S.A.; Yun, S.J.; Jeon, S.H.; Kim, N.H.; Kim, H.W.; Cho, T.J.; Lee, S.H.; Hwang, I.G.; Rhee, M.S. Microbial composition of turbid rice wine (Makgeolli) at different stages of production in a real processing line. *Food Control* 2013, 53, 1–8. [CrossRef]
3. Bal, J.; Yun, S.-H.; Choi, M.-S.; Yeo, S.-H.; Kim, J.-M.; Kim, D.-H. Pyrosequencing reveals bacterial diversity in Korean traditional wheat-based nuruk. *J. Microbiol. Biotechnol.* 2015, 53, 812–819. [CrossRef] [PubMed]
4. Bal, J.; Yun, S.-H.; Yeo, S.-H.; Kim, J.-M.; Kim, D.-H. Metagenomic analysis of fungal diversity in Korean traditional wheat-based fermentation starter nuruk. *Food Microbiol.* 2016, 60, 73–83. [CrossRef]
5. Chai, C.; Lim, G.S.; Kim, Y.J.; Oh, S.W. Microbial community changes in Makgeolli during brewing. *J. Inst. Brew.* 2015, 121, 304–308. [CrossRef]
6. Lee, T.-S.; Choi, J.-Y. Volatile flavor components in Takju fermented with mashed glutinous rice and barley rice. *Korean J. Food Sci. Technol.* 1998, 30, 638–643.
7. Lee, T.-S.; Choi, J.-Y. Volatile Flavor Components in Mash of Takju prepared by using Aspergillus kawachii Nuruk. *Korean J. Food Sci. Technol.* 2005, 37, 944–950.
8. Jung, H.; Lee, S.-J.; Lim, J.H.; Kim, B.-K.; Park, K.J. Chemical and sensory profiles of makgeolli, Korean commercial rice wine, from descriptive, chemical, and volatile compound analyses. *Food Chem.* 2014, 152, 624–632. [CrossRef] [PubMed]
9. Song, Y.-R.; Jeong, D.-Y.; Baik, S.-H. Effects of indigenous yeasts on physicochemical and microbial properties of Korean soy sauce prepared by low-salt fermentation. *Food Microbiol.* 2015, 51, 171–178. [CrossRef] [PubMed]
10. Izco, J.M.; Torre, P. Characterisation of volatile flavour compounds in Roncal cheese extracted by the ‘purge and trap’ method and analysed by GC–MS. *Food Chem.* 2000, 70, 409–417. [CrossRef]
11. Hoenicke, K.; Simat, T.J.; Steinhart, H.; Christoph, N.; Geßner, M.; Köhler, H.-J. ‘Untypical aging off-flavor’ in wine: Formation of 2-aminoacetophenone and evaluation of its influencing factors. *Anal. Chim. Acta* 2002, 458, 29–37. [CrossRef]
12. Jung, M.-J.; Nam, Y.-D.; Roh, S.W.; Bae, J.-W. Unexpected convergence of fungal and bacterial communities during fermentation of traditional Korean alcoholic beverages inoculated with various natural starters. *Food Microbiol.* 2012, 30, 112–123. [CrossRef] [PubMed]
13. Song, S.H.; Lee, C.; Lee, S.; Park, J.M.; Lee, H.J.; Bai, D.H.; Yoon, S.S.; Choi, J.B.; Park, Y.S. Analysis of microflora profile in Korean traditional nuruk. *J. Microbiol. Biotechnol.* 2013, 23, 40–46. [CrossRef] [PubMed]
14. Chi, Z.; Chi, Z.; Liu, G.; Wang, F.; Ju, L.; Zhang, T. *Saccharomycopsis fibuligera* and its applications in biotechnology. *Biotechnol. Adv.* 2009, 27, 423–431. [CrossRef] [PubMed]

15. Styger, G.; Prior, B.; Bauer, F. Wine flavor and aroma. *J. Ind. Microbiol. Biotechnol.* 2011, 38, 1145–1159. [CrossRef] [PubMed]

16. Plata, C.; Millán, C.; Mauricio, J.; Ortega, J. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiol.* 2003, 20, 217–224. [CrossRef]

17. Kang, B.-S.; Lee, J.-E.; Park, H.-J. Qualitative and Quantitative Prediction of Volatile Compounds from Initial Amino Acid Profiles in Korean Rice Wine (*makgeolli*) Model. *J. Food Sci.* 2014, 79, C1106–C1116. [CrossRef] [PubMed]

18. Chuenchomrat, P.; Assavanig, A.; Lertsiri, S. Volatile flavour compounds analysis of solid state fermented Thai rice wine (Ou). *ScienceAsia* 2008, 34, 199–206. [CrossRef]

19. Park, H.-J.; Lee, S.M.; Song, S.H.; Kim, Y.-S. Characterization of Volatile Components in Makgeolli, a Traditional Korean Rice Wine, with or without Pasteurization, During Storage. *Molecules* 2013, 18, 5317–5325. [CrossRef] [PubMed]

20. Dragone, G.; Mussatto, S.I.; Oliveira, J.; Teixeira, J.A. Characterisation of volatile compounds in an alcoholic beverage produced by whey fermentation. *Food Chem.* 2009, 112, 929–935. [CrossRef]

21. Lasekan, O.; Buettner, A.; Christlbauer, M. Investigation of important odorants of palm wine (*Elaeis guineensis*). *Food Chem.* 2007, 105, 15–23. [CrossRef]