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# Development of Korean Indigenous Low-Temperature-Tolerant Yeast for Enhancing the Flavor of *Yakju*

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**Abstract:** *Yakju*, a traditional fermented beverage in Korea, is prepared using various raw materials and methods, and, hence, exhibits various characteristics. Low-temperature-fermented *yakju* can inhibit the growth of undesirable bacteria and is known for its unique flavor and refreshing taste. To increase the production of volatile aromatic compounds in *yakju*, strains with strong resistance to low temperatures and excellent production of volatile aromatic compounds were screened from indigenous fruits (grape, persimmon, plum, aronia, wild grape) and *nuruk* in Korea. One *Saccharomyces cerevisiae* and three non-*Saccharomyces* strains were finally screened, and *yakju* was fermented at 15 °C through mono/co-culture. The analysis of volatile aromatic compounds showed that *S. cerevisiae* W153 produced 1.5 times more isoamyl alcohol than the control strain and reduced the production of 2,3-butanediol by a third. Similarly, a single culture of *Pichia kudriavzevii* N373 also produced 237.7 mg/L of ethyl acetate, whereas *Hanseniaspora vineae* G818 produced ~11 times greater levels of 2-phenethyl acetate than the control. Alternatively, *Wickerhamomyces anomalus* A159 produced 95.88 mg/L of ethyl hexadecanoate. During principal component analysis, we also observed that the co-culture sample exhibited characteristics of both volatile aroma compounds of the single cultured sample of each strain. Our results suggest that *yakju* with unique properties can be prepared using various non-*Saccharomyces* strains.

**Keywords:** *yakju*; low-temperature fermentation; non-*Saccharomyces*; co-culture; volatile aromatic compound



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## 1. Introduction

*Yakju* is a genre of traditional Korean fermented liquor fermented with rice as the primary ingredient. It is characterized by a clean and intrinsic flavor by removing solids through filtration at the end of fermentation. *Yakju* is fermented between 20 and 25 °C, and when the fermentation temperature is lowered, the pH is reduced to suppress harmful bacteria that spoil the liquor; organic acids and volatile aromatic compounds are also different from those produced by fermentation at room temperature [1]. Low-temperature fermentation prevents the loss of primary aromatic compounds and increases the synthesis of secondary aromatic compounds (ethyl and acetate esters) [2–4]. Furthermore, studies have reported that yeast in a low-temperature fermentation environment is influenced by several factors, such as protein transcription, cell membrane fluidity, RNA structure stability, and enzyme activity, as well as aroma component-related reactions [5,6]; therefore, when low-temperature fermentation is performed using a low-temperature-resistant strain, it is possible to prevent spoilage and create *yakju* with a unique profile [7].

The following microorganisms are involved in the fermentation of *yakju*: for saccharification of rice, fungi such as *Aspergillus*, *Rhizopus*, and *Mucor* produce amylase to decompose starch into small molecules of saccharides [8], and then *Saccharomyces cerevisiae* and other

microorganisms work to produce ethanol and numerous organic substances [9,10]. Depending on the yeast that is involved in the fermentation, the result shows significant differences, and the overall preference is affected because of its influence on the senses [11,12].

It is difficult to use most non-*Saccharomyces* strains as ethanol-producing strains in liquor fermentation because of their high sensitivity to ethanol. Moreover, non-*Saccharomyces* yeast has low primary metabolic efficiency, which limits fermentation performance; however, it can affect the organoleptic properties of the final fermentation product due to secondary metabolism [13,14]. It is often used alone or through co-fermentation with *S. cerevisiae* for liquor production [11,13,15,16]. To date, non-*Saccharomyces* strains, such as *Torulaspora* spp. [15,17], *Metschnikowia* spp. [18,19], *Pichia* spp. [20], *Hanseniaspora* spp. [21–23], and *Brettanomyces* spp. have been investigated. Fermentation performed using controlled inoculum can improve the quality of the final product and can ensure better wine quality by utilizing multiple metabolic pathways of non-*Saccharomyces* yeast [24]. Some yeasts have killer activity against spoiled microorganisms, which can be a biological inhibitor to prevent contamination by external microorganisms during fermentation [25,26].

In this study, to improve the flavor of low-temperature-fermented *yakju*, strains with excellent low-temperature tolerance and volatile aromatic compound production ability were first selected among ~700 strains of yeast derived from native fruits and *nuruk* (a traditional Korean fermentation starter). Then, using the selected strain, *yakju* fermentation at 15 °C (single/co-culture) was performed, and the characteristics were analyzed.

## 2. Materials and Methods

### *2.1. Strains and Media*

A total of 661 isolates were used in this experiment, which were isolated from indigenous fruits (persimmon, wild grape, grape, aronia, plums) and *nuruk* owned by the Microbial Biotechnology Laboratory, Department of Food Science and Biotechnology, Kyungpook National University (Daegu, Korea). All strains were subcultured twice at 30 °C by shaking (150 rpm) in sterilized yeast extract peptone dextrose (YPD) media (1% yeast extract, 2% peptone, and 2% glucose (*w*/*v*)). Then, a lysine medium (Oxoid ltd, Basingstoke, Hampshire, UK) was used to confirm the growth of non-*Saccharomyces* strains. All strains were stored at −70 °C in 10% glycerol until they were used for the experiments. *S. cerevisiae* W-3, an industrial wine yeast strain, was used as a control for all experiments.

### *2.2. Screening of Yakju Yeast*

#### 2.2.1. Low-Temperature Fermentation Activity

After inoculation in YPD broth medium, the strains were cultured at 15 °C for 48 h, after which the absorbance was measured at 600 nm using a spectrophotometer (UV-1601, Shimadzu Co., Kyoto, Japan) to select strains with excellent growth. Subsequently, a strain possessing excellent fermentation ability was first screened through its gas-producing ability and sniffing tests performed by culturing in a rice saccharification solution at 15 °C for 96 h. In the case of the sniffing test, it was classified into positive and negative according to the occurrence of an off-flavor.

#### 2.2.2. Screening by Internal Transcribed Spacer (ITS) Region Sequencing

The identification of the first screened strain was performed through phylogenetic analysis based on the nucleotide sequence of the ITS I-5.8S rDNA-ITS II region. After DNA extraction using the method by Looke et al. [27], ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers were then used with a Tpersnal 48 PCR machine (Biometra Co., Göttingen, Germany) for amplification. Then, for PCR-RFLP, *Hinf I*, *Hae III*, and *Hpa I* (Enzynomics Co., Daejeon, Korea) restriction enzymes were reacted at 37 °C for 1 h, after which DNA fragments were identified through electrophoresis. DNA sequencing was performed using the sequencing service of Solgent (Daejeon, Korea), and the nucleotide sequence homology was checked using the Basic Local

Alignment Search Tool of the National Center for Biotechnology Information. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura, Stecher, Peterson, Filipski, and Kumar, 2013).

#### 2.2.3. β-Glucosidase Enzyme Activity

A modified Rodríguez's method was used for measuring β-glucosidase activity [28]. The first screened strain was incubated for 48 h in YPD broth, and 1 mL was collected and centrifuged (1536, GYROZEN Co., Daejeon, Korea) at 9425× *g* for 3 min. Next, 0.2 mL of the supernatant, 0.2 mL of 20 mM pNPG (ρ-nitrophenyl-β-D-glucopyranoside), and 0.4 mL of 0.1 M citrate phosphate buffer (pH 5.0) were mixed and reacted in a water bath at 40 °C for 30 min. Finally, 0.8 mL of 2 M sodium carbonate was added to terminate the reaction, and absorbance was measured at 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol pNPG per minute under the experimental conditions.

#### 2.2.4. Biosynthesis Inhibitor Resistance Activity

Cerulenin agar (0.67% yeast nitrogen base, 0.5% 10 mM cerulenin, 2% glucose, and 2% agar) and FPA agar (0.67% yeast nitrogen base, 0.4% 1 M FPA (ρ-fluoro phenylalanine), 2% glucose, and 2% agar) were inoculated with a single colony of the first screened strain. Resistance was confirmed by colony formation after incubation for 48 h at 30 °C [29].

### *2.3. Analysis of Low-Temperature-Fermented Yakju*

#### 2.3.1. Brewing *Yakju*

Steamed rice 270 g (Shindongjin, Iksan Rice Processing Plant, Iksan, Jeollabuk-do, Korea), *koji* 50 g (*Aspergillus luchuensis*, sp 60, Cheokgokshik, Hwaseong, Gyeonggi-do, Korea), 480 mL water, and 2.5% of the total volume of pre-cultured yeast were mixed. It was mixed once a day while fermentation was proceeding at 15 °C. For co-fermented samples, pre-cultured *S. cerevisiae* and non-*Saccharomyces* were mixed in a ratio of 1:9 and inoculated simultaneously. Growth of *S. cerevisiae* and non-*Saccharomyces* in co-fermented samples was compared every 5 days using Lee's method [30]. Fermentation was terminated when no change was observed in the content of reducing sugars as assessed using colorimetry. Finally, all samples were centrifuged at 3578× *g* for 10 min and stored at 4 °C for further experiments.

#### 2.3.2. Fermentation Characteristics of *Yakju*

The pH value was measured using an automatic titration device (DL22 Food and Beverage Analyzer, Mettler-Toledo AG Analytical, Schwerzenbach, Switzerland). Soluble solid content (° Brix) was measured using a refractometer (ATAGO, Co., N-1a, Kyoto, Japan). The reducing sugar content was measured by colorimetric quantification using DNS (3,5-dinitrosalicylic acid, Sigma Chemical Co., St. Louis, MO, USA) reagent. After adding 1 mL of DNS reagent to 0.3 mL of a sample to induce a reaction at 95 °C for 5 min, 7 mL of distilled water was added to measure the absorbance at 550 nm. The reducing sugar content was converted from the glucose standard curve. The alcohol content was measured using a hydrometer based on the specific gravity of *yakju* distillates (expressed as % [*v*/*v*]) at 15 °C.

#### 2.3.3. Volatile Aromatic Compound Analysis by Gas Chromatography

The volatile aromatic compounds were determined using a gas chromatography mass spectrometer (7890A GC-MS; Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector, and analysis methods were referred to previously described methods [31]. The separation was performed using a DB-WAX column (60 m × 250 μm × 0.25 mm; Waters, Milford, MA, USA). The detector was an Agilent 5975C Inert XL MSD with a Triple-Axis Detector. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The temperature of the chromatographic oven was initially held at 40 °C for 2 min, increased at

2 °C/min to 220 °C, increased continuously at 20 °C/min to 240 °C, and then maintained at 240 °C for 5 min. The volatile ester compounds were collected using a solid-phase microextraction fiber (50/30 μm DVB/CAR/PDMS; Supelco, Bellefonte, PA, USA). Extraction of the volatile ester compounds from *yakju* was performed in a headspace (HS) mode with magnetic stirring. Briefly, 5 mL of the sample was placed in an HS vial (20 mm, PTFE/silicone septum, magnetic cap), and 1.25 g of NaCl was added to increase the volatile ester compound concentration in the HS. Before extraction, the sample was shaken in a water bath at 35 °C for 20 min to achieve equilibrium. The volatile ester compounds were identified based on a comparison of their GC retention times and mass spectrometry with spectral data from the Wiley 9th Edition/Nist 2008 library program(Wiley 9th Edition/Nist 2008 library version 5.0; John Wiley & Sons Inc., Hoboken, NJ, USA).

### *2.4. Statistical Analysis*

Data were expressed as the mean ± standard deviation (SD) of three experiments. The SAS program (Statistics Analysis System, SAS Institute Inc., Cary, NC, USA) was used to perform analysis of variance and Duncan's multi-range testing. The threshold level for statistical significance was set at $p < 0.05$. Principal component analysis (PCA) was conducted using R studio's factoextra (https://cran.r-project.org/web/packages/factoextra/index.html, accessed on 6 November 2021) and ggplot2 (https://cran.r-project.org/web/packages/ggplot2/index.html, accessed on 6 November 2021).

## 3. Results and Discussion

### *3.1. Screening of Yakju Strains*

The results of the screening of strains are expressed in Tables S1 and S2. Through the first screening (Table S1), 79 strains with excellent low-temperature growth and sniffing test results were first selected. Based on the result of ITS region nucleotide sequencing, 22 *S. cerevisiae* and 57 non-*Saccharomyces* were identified. Among them, there were 40 *Wickerhamomyces anomalus*, 12 *Hanseniaspora vineae*, 3 *Torulaspora delbrueckii*, and 2 *Pichia kudriavzevii* strains. Then, the biosynthesis inhibitor resistance test and β-glucosidase enzyme activity experiment were conducted, and the results are shown in Supplementary Table S2 (Table S2). The β-glucosidase enzyme activity was higher than 1 U/mL in 11 strains, and in the case of *P. kudriavzevii* N373, 1.41 U/mL was the highest activity. In the case of resistance to FPA, most *H. vineae* strains showed high resistance (11/12), and *P. kudriavzevii* also demonstrated the same result in both strains (2/2). For cerulenin resistance, colonies were identified in *W. anomalus* (39/40), *T. delbreuckii* (3/3), and *P. kudriavzevii* (2/2).

As β-glucosidase hydrolyzes β-1,4 bonds of nonvolatile glycosidic compounds to release volatile aromatic compounds [32,33], strains with high β-glucosidase enzyme activity can produce a large amount of volatile aromatic compounds include 2-phenethyl alcohol. Cerulenin is an antibiotic that inhibits the biosynthesis of fatty acids. The higher the resistance to cerulenin, the more favorable it is to produce medium-chain fatty acid (MCFA) ethyl esters, especially ethyl caproate, a flavor representing traditional Korean liquor, which can be produced up to five times [34]. ρ-Fluoro-phenylalanine (FPA) is a biosynthesis inhibitor through feedback and exists in the form of fluoroalanine attached to phenylalanine. The stronger the resistance to this compound, the more it produces 2-phenylethanol and 2-phenylethyl acetate, which gives off a rose-like scent [29].

Through the two-step screening, one *S. cerevisiae* and three non-*Saccharomyces* strains were finally selected, and one isolate from each strain was selected for the diversification of fragrance components, as shown in Table 1.

*S. cerevisiae* W153 exhibited the best cell growth at 15 °C, and *P. kudriavzevii* N373 was resistant to both cerulenin and FPA. *H. vineae* G818 and *W. anomalus* A159 strains exhibited resistance to FPA and cerulenin, respectively.

**Table 1.** List of final selected strains.

| Strains | Species | Cell Growth ($OD_{600nm}$, 15 °C) | β-Glucosidase Activity (U/mL) | Cerulenin Resistance | FPA Resistance |
|---|---|---|---|---|---|
| W153 | *Saccharomyces cerevisiae* | 1.615 | 0.679 | + | − |
| N373 | *Pichia kudriavzevii* | 1.285 | 1.408 | + | + |
| G818 | *Hanseniaspora vineae* | 0.977 | 0.567 | − | + |
| A159 | *Wickerhamomyces anomalus* | 1.161 | 0.922 | + | − |

### 3.2. Characteristics of Yakju

The characteristics of *yakju* after fermentation are shown in Figure 1. In the case of the co-culture samples, the growth of each strain was confirmed by the viable cell count and is shown in Supplementary Figure S1 (Figure S1, the results of the single cultures are not shown). The co-culture with non-*Saccharomyces* strains showed the lowest pH value of 3.32–3.35 (Figure 1a). The soluble solid content was at the maximum (10.37 Brix°) in *W. anomalus* A159, and *H. vineae* G818 showed the highest reducing sugar content (Figure 1b,c). *S. cerevisiae* single/co-culture samples consumed all the reducing sugars, whereas the non-*Saccharomyces* group did not, with the expectation of a decrease or death occurring as the ethanol content increased during fermentation [35]. Because most non-*Saccharomyces* species had lower ethanol resistance and titers than those of *S. cerevisiae* [36], the final ethanol content was also lower than that of *S. cerevisiae*. Nevertheless, all fermented samples showed a high ethanol content of 8% or more, which was expected to be characteristic of low-temperature long-term fermentation and *yakju* [7,37].

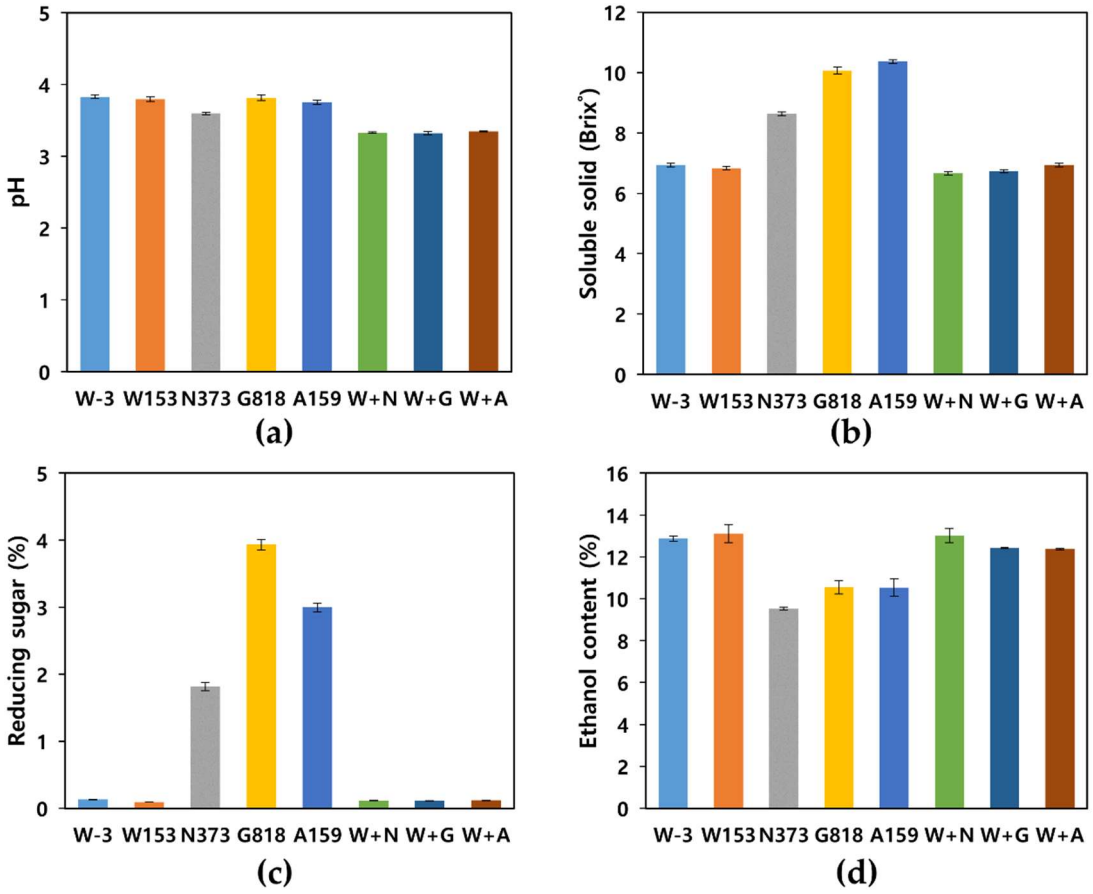


**Figure 1.** Characteristics of *yakju* after fermentation: (**a**) pH; (**b**) Soluble solid (Brix°); (**c**) Reducing sugar (%); (**d**) Ethanol content (%). **W + N**, co-culture of W153 + N373; **W + A**, co-culture of W153 + A159; **W + G**, co-culture of W153 + G818.

In the case of the co-culture group (W153 + N373, W153 + G818, and W153 + A159), an amount of ethanol similar to that of the single culture of *S. cerevisiae* was produced (Figure 1d), which was consistent with the results of previous studies [36,38,39]. The *S. cerevisiae* W153 strain exhibited fermentation characteristics similar to those of the control.

### 3.3. Volatile Aromatic Compounds

Regarding the volatile aromatic compounds of *yakju*, six alcohols and fourteen esters were detected by headspace GC-MS, as shown in Table 2. Compared with the results obtained from the analysis of volatile aroma components of commercial *yakju* [40], a large concentration of higher alcohols and esters was produced. This high concentration was expected due to differences in fermentation processes (dilution) and conditions (temperature, fermentation time, etc.). In the single culture, *S. cerevisiae* (W-3 and W153) produced more total higher alcohol content (507–556 mg/L) than non-*Saccharomyces* strains (206–397 mg/L), whereas regarding esters, non-*Saccharomyces* strains produced two to three times higher ester content. In the case of co-culture, the characteristics of the strains used for fermentation appeared similar, and the content of total volatile aromatic compounds increased. Some components showed higher values than those of single fermentation (isoamyl alcohol, 1-propanol, 2-phenethyl alcohol, and ethyl decanoate).

**Table 2.** Volatile aromatic compounds of *yakju* after fermentation.

| Volatile Compounds | W-3 | W153 | N373 | G818 | A159 | W153 + N373 | W153 + G818 | W153 + A159 |
|---|---|---|---|---|---|---|---|---|
| Higher alcohols (mg/L) | | | | | | | | |
| 1-Propanol | 30.40 ± 0.33 $^{b(1)(2)}$ | 6.82 ± 0.19 $^{g}$ | 10.01 ± 0.62 $^{f}$ | 21.41 ± 0.31 $^{d}$ | 6.78 ± 0.45 $^{g}$ | 28.38 ± 1.13 $^{c}$ | 48.46 ± 0.86 $^{a}$ | 17.12 ± 0.36 $^{e}$ |
| Iso-butanol | 241.22 ± 10.90 $^{a}$ | 144.52 ± 4.81 $^{c}$ | 83.94 ± 0.64 $^{e}$ | 39.25 ± 2.84 $^{f}$ | 25.09 ± 0.98 $^{g}$ | 181.01 ± 7.40 $^{b}$ | 146.87 ± 6.87 $^{c}$ | 114.08 ± 3.78 $^{d}$ |
| Isoamyl alcohol | 158.89 ± 8.89 $^{d}$ | 245.08 ± 5.25 $^{b}$ | 200.81 ± 8.18 $^{c}$ | 196.14 ± 9.31 $^{c}$ | 110.76 ± 6.46 $^{e}$ | 204.56 ± 5.63 $^{c}$ | 262.37 ± 6.86 $^{a}$ | 48.93 ± 5.12 $^{f}$ |
| Methionol | 2.89 ± 0.77 $^{d}$ | 4.19 ± 0.29 $^{c}$ | 10.92 ± 0.39 $^{a}$ | 3.03 ± 0.52 $^{d}$ | 8.26 ± 0.86 $^{b}$ | 4.69 ± 0.93 $^{c}$ | 4.43 ± 0.27 $^{c}$ | 7.67 ± 0.69 $^{b}$ |
| 2-Phenetyl alcohol | 85.44 ± 8.63 $^{cd}$ | 95.92 ± 6.22 $^{ab}$ | 76.80 ± 4.08 $^{d}$ | 27.21 ± 2.18 $^{f}$ | 42.16 ± 1.60 $^{e}$ | 87.86 ± 6.16 $^{bc}$ | 80.54 ± 3.58 $^{cd}$ | 104.26 ± 6.24 $^{a}$ |
| 2,3-Butanediol | 36.75 ± 1.79 $^{a}$ | 11.35 ± 0.95 $^{ef}$ | 14.34 ± 1.63 $^{d}$ | 10.13 ± 0.62 $^{f}$ | 12.58 ± 0.28 $^{de}$ | 13.55 ± 0.77 $^{d}$ | 18.95 ± 0.96 $^{c}$ | 25.72 ± 1.59 $^{b}$ |
| Esters (mg/L) | | | | | | | | |
| Ethyl acetate | 26.08 ± 2.69 $^{e}$ | 28.29 ± 3.19 $^{ef}$ | 237.70 ± 28.8 $^{a}$ | 115.95 ± 7.31 $^{b}$ | 71.74 ± 8.14 $^{cd}$ | 85.72 ± 7.17 $^{c}$ | 56.62 ± 5.13 $^{d}$ | 61.76 ± 3.74 $^{d}$ |
| Isobutyl acetate | 8.41 ± 0.67 $^{a}$ | 9.17 ± 0.77 $^{a}$ | 2.88 ± 0.35 $^{c}$ | 1.06 ± 0.16 $^{d}$ | 2.06 ± 0.30 $^{cd}$ | 9.07 ± 1.32 $^{a}$ | 5.45 ± 0.54 $^{b}$ | 7.97 ± 1.22 $^{a}$ |
| Ethyl butyrate | 0.42 ± 0.03 $^{a}$ | 0.40 ± 0.02 $^{ab}$ | 0.17 ± 0.02 $^{d}$ | 0.09 ± 0.01 $^{e}$ | 0.21 ± 0.02 $^{d}$ | 0.42 ± 0.02 $^{a}$ | 0.36 ± 0.01 $^{bc}$ | 0.33 ± 0.06 $^{c}$ |
| Isoamyl acetate | 10.62 ± 1.56 $^{a}$ | 9.74 ± 0.43 $^{ab}$ | 7.20 ± 2.03 $^{c}$ | 4.04 ± 0.36 $^{d}$ | 0.85 ± 0.17 $^{e}$ | 8.12 ± 1.27 $^{bc}$ | 4.60 ± 0.57 $^{d}$ | 5.02 ± 0.16 $^{d}$ |
| Ethyl hexanoate | 0.50 ± 0.02 $^{b}$ | 0.54 ± 0.01 $^{a}$ | 0.10 ± 0.03 $^{e}$ | 0.07 ± 0.01 $^{ef}$ | 0.05 ± 0.01 $^{f}$ | 0.49 ± 0.02 $^{b}$ | 0.30 ± 0.01 $^{d}$ | 0.35 ± 0.03 $^{c}$ |
| Ethyl octanoate | 1.36 ± 0.22 $^{a}$ | 1.39 ± 0.08 $^{a}$ | 0.16 ± 0.13 $^{d}$ | 0.17 ± 0.04 $^{d}$ | 0.06 ± 0.02 $^{d}$ | 1.27 ± 0.11 $^{a}$ | 0.81 ± 0.12 $^{c}$ | 1.05 ± 0.09 $^{b}$ |
| Ethyl nonanoate | 5.84 ± 0.32 $^{e}$ | 3.81 ± 0.33 $^{f}$ | 7.17 ± 0.41 $^{e}$ | 11.54 ± 1.12 $^{c}$ | 15.88 ± 0.35 $^{a}$ | 8.75 ± 1.12 $^{d}$ | 10.60 ± 1.36 $^{c}$ | 12.98 ± 0.81 $^{b}$ |
| Ethyl decanoate | 26.05 ± 1.02 $^{ab}$ | 22.73 ± 1.31 $^{c}$ | 4.04 ± 0.47 $^{e}$ | 19.37 ± 1.39 $^{d}$ | 1.80 ± 0.08 $^{f}$ | 23.53 ± 1.35 $^{c}$ | 28.08 ± 1.98 $^{a}$ | 23.99 ± 1.19 $^{bc}$ |
| Diethyl succinate | 2.59 ± 0.36 $^{ab}$ | 2.26 ± 0.15 $^{bc}$ | 0.40 ± 0.06 $^{d}$ | 1.93 ± 0.27 $^{c}$ | 0.18 ± 0.05 $^{d}$ | 2.34 ± 0.24 $^{b}$ | 2.80 ± 0.09 $^{a}$ | 2.39 ± 0.08 $^{b}$ |
| Ethyl dodecanoate | 2.63 ± 0.15 $^{d}$ | 1.74 ± 0.09 $^{ef}$ | 1.60 ± 0.11 $^{f}$ | 2.08 ± 0.09 $^{e}$ | 1.49 ± 0.08 $^{f}$ | 3.17 ± 0.18 $^{c}$ | 4.41 ± 0.27 $^{a}$ | 3.74 ± 0.48 $^{b}$ |
| Methyl salicylate | 1.43 ± 0.18 $^{c}$ | 1.48 ± 0.07 $^{c}$ | 1.65 ± 0.16 $^{bc}$ | 2.08 ± 0.17 $^{a}$ | 2.05 ± 0.24 $^{a}$ | 1.40 ± 0.16 $^{c}$ | 1.71 ± 0.19 $^{bc}$ | 1.83 ± 0.15 $^{ab}$ |
| 2-Phenethyl acetate | 3.18 ± 0.28 $^{de}$ | 5.77 ± 0.35 $^{c}$ | 1.59 ± 0.26 $^{ef}$ | 33.76 ± 1.80 $^{a}$ | 0.26 ± 0.02 $^{f}$ | 4.08 ± 0.94 $^{cd}$ | 28.38 ± 1.97 $^{b}$ | 0.75 ± 0.09 $^{f}$ |
| Ethyl tetradecanoate | 2.66 ± 0.13 $^{fg}$ | 2.38 ± 0.33 $^{g}$ | 3.39 ± 0.27 $^{e}$ | 8.44 ± 0.45 $^{b}$ | 10.51 ± 0.51 $^{a}$ | 3.07 ± 0.28 $^{ef}$ | 5.61 ± 0.07 $^{c}$ | 4.66 ± 0.38 $^{d}$ |
| Ethyl hexadecanoate | 10.39 ± 1.26 $^{g}$ | 14.78 ± 0.96 $^{f}$ | 17.78 ± 2.47 $^{f}$ | 38.00 ± 2.33 $^{d}$ | 95.88 ± 1.07 $^{a}$ | 29.40 ± 2.39 $^{e}$ | 43.76 ± 1.48 $^{c}$ | 50.65 ± 1.93 $^{b}$ |

(1) All data were expressed as mean ± SD (n = 3); (2) Different letters within the same column indicate significant differences ($p < 0.05$).

Higher alcohols were quantitatively the most abundant group in *yakju* and contributed to the aroma and essential characteristics [40]. However, excessive production of higher alcohols has a negative impact on the aroma, and an appropriate concentration can have a positive impact, such as a fruity flavor [41]. Isoamyl alcohol is a major volatile compound among higher alcohols, which boosts the aroma and flavor [42]. *S. cerevisiae* W153 produced ~1.5 times more isoamyl alcohol than the control strain (*S. cerevisiae* W-3). One of the primary components of wine, 2,3-Butanediol, is produced from carbohydrates during the alcohol fermentation process and has a negative impact on liquor by causing bitter taste,

vinegar odor, and yeast odor [43,44]. *S. cerevisiae* W153 could reduce the production of 2,3-butanediol by one-third. Ethyl acetate levels with higher quantities of 150 mg/L are a measure of non-*Saccharomyces*-dependent spoilage in the wine industry [45]. However, a large quantity of ethyl acetate is preferred for grain-based alcoholic beverages and spirits [46,47]. During this study, *P. kudriavzevii* N373 produced approximately nine times more ethyl acetate (237 mg/L) than the control.

MCFA ethyl esters (ethyl hexanoate, ethyl octanoate, ethyl nonanoate, and ethyl decanoate) are fatty acid ethyl esters composed of 6–12 carbon atoms and have a low threshold, which can cause a large sensory change even in a small amount [48]. Particularly, ethyl hexanoate (ethyl caproate) is an important fragrance ingredient in products such as premium sake and exhibits a pleasant, fruity apple-like flavor [49]. Two *S. cerevisiae* strains (W-3, W153) produced the highest amount of ethyl hexanoate (0.50–0.54 mg/L) [50], and co-culture increased the production compared to that with a non-*Saccharomyces* single culture. In the case of ethyl nonanoate, *W. anomalus* A159 showed the highest value of 11.54. The production of 2-phenylethyl acetate by yeast cells is responsible for a fruity, flowery flavor. *H. vineae* G818, FPA biosynthesis-resistant-positive strain, produced ~33.76 mg/L of 2-phenethyl acetate, which was ~11 times higher than that of the control and co-culture with *S. cerevisiae* W153 also demonstrated similar results (28.38 mg/L) [51]. Ethyl tetradecanoate (ethyl myristate) and ethyl hexadecanoate (ethyl palmitate), components of aged whiskey [52], were four times and nine times higher, respectively, in a single culture of *W. aomalus* A159.

### *3.4. Principal Component Analysis*

Figure 2 shows the biplot results for the volatile fragrance components. Based on PC1, it could be divided into two groups: non-*Saccharomyces* single culture (B) and *S. cerevisiae* single/co-culture samples (A). Moreover, it was confirmed that PC2 showed similar values depending on the non-*Saccharomyces* strain involved in fermentation. In the case of *P. kudriavzevii* N373, a significant difference was observed between the single culture and co-culture (W + N, N373), but the single fermentation of A159 and G818 strains showed similar PC2 values to those of co-culture (W + A and W + G, respectively). Results of the loading plot (blue arrow) indicate that most aromatic compounds were contained in group A (13/20), and the aromatic compounds were strengthened through co-culture. Methionol, ethyl acetate, methyl salicylate, and ethyl tetradecanoate were specifically produced in large amounts in the single-culture non-*Saccharomyces* group.

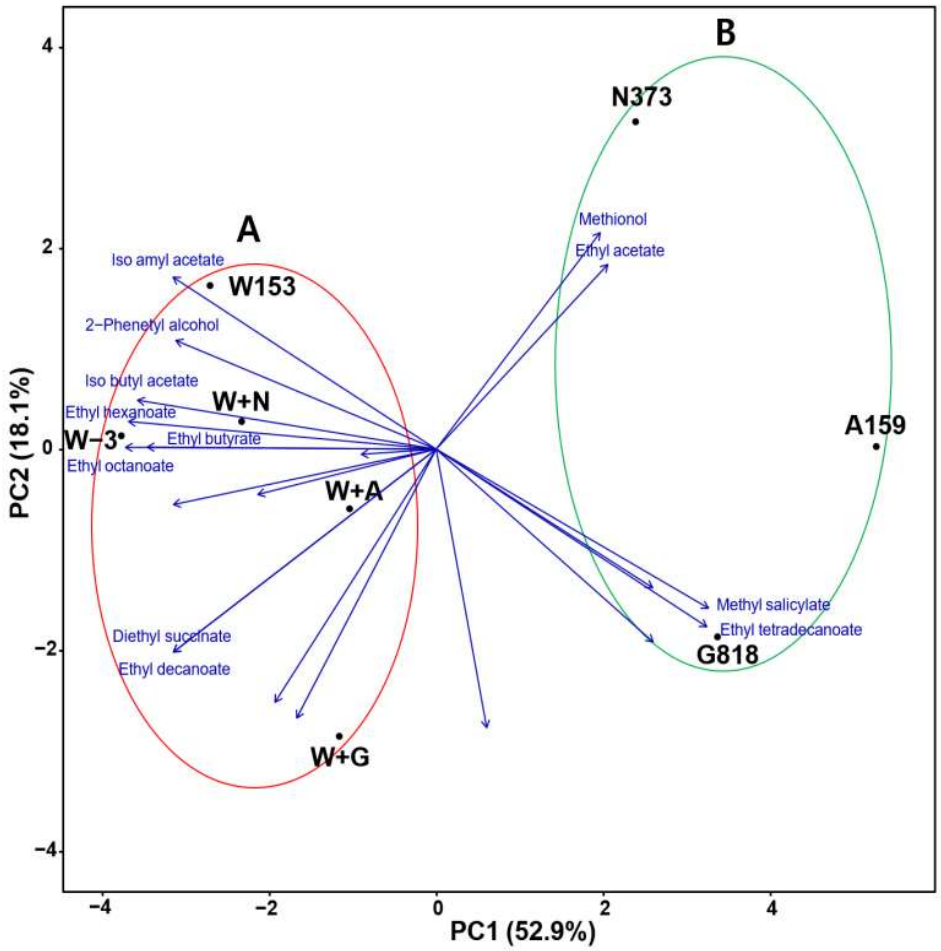


**Figure 2.** Biplot of variable loadings (represented by vectors) and scores (marked by points) from the principal component analysis (PCA) of volatile fragrance. **W + N**, co-culture of W153 + N373; **W + A**, co-culture of W153 + A159; **W + G**, co-culture of W153 + G818.

## 4. Conclusions

Low-temperature fermentation prevents bacteria-dependent spoilage and preserves flavor; therefore, this method produces a different aroma component profile from that observed with room temperature fermentation. To strengthen the flavor components of *yakju*, a traditional Korean alcoholic beverage, strains possessing excellent low-temperature fermentation abilities, and which could produce numerous volatile aromatic compounds were selected from indigenous fruits and *nuruk*. Its fermentation characteristics were then compared after the preparation of low-temperature *yakju*. In this study, *yakju* was prepared using selected strains, which were mono or co-cultured with *Saccharomyces cerevisiae* and fermented at 15 °C. Due to the analysis of volatile aroma components produced, characteristic esters and higher alcohols were detected in each strain. Therefore, it was confirmed that the co-culture samples had characteristics of both strains. Based on these studies, it was, however, expected that *yakju*, having various aroma component profiles, would be produced using several indigenous yeasts. In addition to *S. cerevisiae*, a traditional yeast used in *yakju* brewing, studies have shown that *yakju*, having a unique aromatic profile, was produced using non-*Saccharomyces* yeast. However, specific correlation studies on the metabolism of the volatile fragrance component products in each strain and profile change assessments according to raw materials are required. Additional studies on changes in the fermentation profile according to the inoculation time and ratio of mixed samples are also required. Through these studies, we propose that it will be possible to contribute to the

localization of *yakju*-based yeast and the manufacture of *yakju* using various volatile aroma components.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/fermentation7040260/s1, Table S1: Low-temperature-tolerant and fermentation activity of isolated strains; Table S2: Biosynthesis inhibitor resistance and enzyme activity of first screened strains; Figure S1: Viable cell number of co-culture strain used in yakju fermentation.

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