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Development of air-blast dried non-*Saccharomyces* yeast starter for improving quality of Korean persimmon wine and apple cider

Da-Hye Kim[a,1], Sae-Byuk Lee[a,1], Jun-Young Jeon[a], Heui-Dong Park[a,b,*]

[a] School of Food Science and Biotechnology, Kyungpook National University, Daegu 41566, South Korea
[b] Institute of Fermentation Biotechnology, Kyungpook National University, Daegu 41566, South Korea

A R T I C L E I N F O

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A B S T R A C T

A total of 512 yeasts, including 422 non-*Saccharomyces* yeasts, were isolated from various fruits including apple, aronia, Muscat Bailey A grapes, and persimmon. These were used to prepare persimmon wine and apple cider starters that produced high levels of aromatic compounds, which contribute to high-quality fermented products. Environmental tolerance testing with 20% glucose and 8% EtOH, alongside a sniffing test, led to the selection of *Wickerhamomyces anomalus* (Synonym *Pichia anomala*) SJ20, *Meyerozyma caribbica* (Synonym *Pichia caribbica*) YP1, *Pichia kluyveri* CD34, *Hanseniaspora uvarum* SJ69 (for persimmon wine), *W. anomalus* CS7-16 (for apple cider), and *Starmerella bacillaris* (Synonym *Candida zemplinina)* CD80 (for both wines) as wine starters. These strains had high environmental stress tolerance and the highest sniffing test scores. Persimmon wine and apple cider were fermented using these strains in single- or mixed-culture with *S. cerevisiae* W-3 to determine the improved effect on wine aroma. In accordance with the results of volatile ester compounds and sensory evaluation, *W. anomalus* SJ20, *H. uvarum* SJ69, and *W. anomalus* CS7-16 had an excellent potential as persimmon wine and apple cider starters. Moreover, other strains also showed a good potential for a distinctive persimmon wine and apple cider because of the different compositions of the various volatile ester compounds. Six types of sugars (fructose, glucose, maltose, sucrose, raffinose, sucrose, and trehalose), four types of rehydration solutions (distilled water, 1× phosphate buffered saline, 0.85% NaCl, and 1% peptone water), and two types of antioxidants (L-ascorbic acid and glutathione) were examined to improve the survival rate of air-blast dried non-*Saccharomyces* yeast cells. Optimal sugar and rehydration conditions for each strain were validated, and scanning electron microscopy showed that each cell was surrounded by protectants, including sugar, skim milk, and lactomil. Storability assessment of air-blast dried yeast cells maintained at 4 °C for two months indicated that at least one condition in each strain had a higher survival rate than the control, regardless of the concentration or type of antioxidant treatment, except for *M. caribbica* YP1. These results suggest that antioxidant treatment contributes to maintaining the viability of air-blast dried cells in hostile environments.

1. Introduction

Yeast has long been utilized in the production of a wide range of materials across many industries, including alcoholic beverages, leavened breads, and pharmaceuticals (Stanley et al., 2010). It is particularly well-known as a main starter in the wine industry because of the high ethanol tolerance compared to other microorganisms (Casey and Ingledew, 1986; Philliskirk and Young, 1975). Similar to other industrial microorganisms, wine yeasts have been produced in a starter powder form that can be easily supplied to wine manufacturers. The majority of Korean winemakers utilize *Saccharomyces cerevisiae* Fermivin from the Netherlands, *S. cerevisiae* W-3 from Japan, or *S. cerevisiae* EC1118 from Canada due to their convenience and reliability (Lee et al., 2016a). While these starters exhibit excellent alcohol producing ability, they have a limitation in accordance with the organoleptic qualities of the wine product, such as those derived from weak volatile aromatic compounds. As an alternative, non-*Saccharomyces* yeasts that grow during the early phases of fermentation have recently been reported as a potential alternate resource to improve wine quality (Contreras et al., 2015; Jolly et al., 2006; Padilla et al., 2016), especially after co-fermentation with *Saccharomyces* yeasts (Kim et al., 2008; Lema et al., 1996; Sadoudi et al., 2012).

To overcome the low competitiveness of Korean grape wine due to the relatively high acidity and weak flavor of local grape cultivars (Kim

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[*] Corresponding author at: School of Food Science and Biotechnology, Kyungpook National University, Daegu 41566, South Korea.
E-mail address: hpark@knu.ac.kr (H.-D. Park).
[1] D-H K and S—B L contributed equally to this work.

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et al., 1999), Korean winemakers have focused on producing wine from other indigenous fruits, such as persimmon and apples (Bae et al., 2002; Han et al., 2009; Kwon et al., 2015). However, because all the wine yeast starters used for persimmon wine and apple cider are based on *S. cerevisiae*, no notable differences in the quality of these products are apparent, aside from those arising from the characteristics of the main ingredient. Conversely, several researchers have reported that indigenous yeasts can generate distinctive wines, depending on the grape cultivar, geographical region, and composition of inoculated target fruits (Heard and Fleet, 1985; Hong and Park, 2013; Mercado et al., 2007; Querol et al., 1992; Schütz and Gafner, 1993). Accordingly, the development of indigenous non-*Saccharomyces* yeasts appropriate for persimmon wine and apple cider may contribute to a more competitive Korean wine industry.

The development of wine yeast starters for persimmon wine and apple cider is necessary for easier application by winegrowers and to facilitate the introduction of indigenous non-*Saccharomyces* yeast starters into the wine industry. For this, various factors such as drying methods, rehydration conditions, and protective agents should be considered. Among the various drying technologies, freeze-drying, a process that is generally used for generating microorganism starters, requires a long manufacturing time and is expensive. Furthermore, fluidized bed and spray drying are unsuitable because of cell damage induced by the high temperatures used (Santivarangkna et al., 2007). As an alternative, we investigated in a previous study whether air-blast drying of *S. cerevisiae* D8, *Hanseniaspora uvarum* S6, and *Issatchenckia orientalis* KMBL5774 yeast starters could resolve the disadvantages incurred by other drying technologies (Lee et al., 2016a). Air-blast drying can reduce the manufacturing cost five-fold compared to freeze-drying and help to easier control of the moisture in the starter with ease, leading to less cell damage compared to fluidized bed drying and spray drying (Lee et al., 2016a; Jenkins et al., 2011; Santivarangkna et al., 2007). In our prior study, lactomil using skim milk as a protective agent was selected as the most suitable excipient for an air-blast dried yeast starter because of shorter drying time, high survival rate, and the generation of a fine powder. In addition, different types of sugar were also investigated for different yeast strains and each strain showed different optimal protective agents (Lee et al., 2016a). In addition to prolonged storability through the addition of an antioxidant that prevents cell membrane oxidation during storage, some researchers have revealed that improved viability can be obtained depending on rehydration conditions such as composition, temperature, and time prior to starter use (Abadias et al., 2001; Jenkins et al., 2011; Peña et al., 1992). Several studies have reported that glutathione and L-ascorbic acid play protective roles against oxidative stress responses in yeast (Carmel-Harel and Storz, 2000; Heick et al., 1972; Izawa et al., 1995). Despite the importance of antioxidants to the viability of microorganism starters, most studies using antioxidants as a protectant have only focused on probiotics (Dave and Shah, 1997; Yao et al., 2008; Zhang et al., 2010).

In this study, we isolated various non-*Saccharomyces* yeasts from various food materials. In addition, various environmental tolerances and sniffing test to develop a non-*Saccharomyces* wine starter to improve the quality of persimmon wine and apple cider were investigated. To verify the aroma forming ability of selected non-*Saccharomyces* yeasts, the strains were inoculated into persimmon and apple juices, and their fermentation characteristics were examined. To select a non-*Saccharomyces* yeast starter for air-blast drying, various experiments were conducted alongside the optimization of protectant and rehydration conditions using different types of sugars, antioxidants, and rehydration solutions to enhance the viability and storability of air-blast dried yeast cells. Furthermore, the fermentation rate of air-blast dried yeast cells was investigated to evaluate the potential of the newly developed yeast starters for industrial application. Together, these studies have led to the identification of potential new non-*Saccharomyces* starters for wine production that could improve quality in Korea and further afield.

2. Materials and methods

2.1. Strains, media, and culture conditions

Wickerhamomyces anomalus SJ20 (KACC93297P), *Hanseniaspora uvarum* SJ69 (KACC93298P), *Pichia kluyveri* CD34 (KACC93292P), *Starmerella bacillaris* CD80 (KACC93299P), *Meyerozyma caribbica* YP1 (KACC93291P), and *W. anomalus* CS7–16 (KACC93290P) were all isolated and used in this study. Each strain was cultured at 30 °C with shaking (150 rpm) in sterilized YPD media containing 1% yeast extract, 2% bacto-peptone, and 2% glucose. Cells were then harvested to create the starters. All strains were stored at −70 °C in 10% glycerol until they were used for the experiments.

2.2. Development of non-Saccharomyces yeasts suitable for persimmon wine and apple cider

2.2.1. Isolation of yeasts

Yeasts were isolated from various fruits prepared by crushing, including 'fuji' apple, aronia, MBA grapes, and persimmon. Crushed fruit mush (100 μL) was plated onto YPD agar media containing 50 μg/mL ampicillin and 0.4% sodium propionate to prevent bacterial and fungal growth and cultured at 30 °C for 24 h. Any colonies that formed on the YPD agar were isolated and their morphology assessed to distinguish individual yeast isolates using a microscope.

2.2.2. Sequence analysis of the internal transcribed spacer (ITS) region of isolated yeasts

All isolates that were considered yeast strains were subjected to PCR-RFLP with amplification of the ITS I-5.8S rDNA-ITS II region. Chromosomal DNA for the PCR template was isolated from yeast cells grown in YPD media for 24 h using a method described by Kaiser et al. (1994) and Philippsen et al. (1991). The primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) used for PCR were synthesized by Bioneer Co. (Chongwon, Korea). PCR was performed in a 20 μL reaction volume using TaKaRa Taq DNA polymerase (TaKaRa Shuzo Co., Otsu, Japan) with a GENE cycler (BioRad Co., Richmond, VA, USA). The PCR mix consisted of 1 μg yeast genomic DNA, 100 pmol of each primer, 1 U Taq DNA polymerase, and 0.25 mM $MgCl_2$. The PCR cycling program for DNA amplification consisted of one cycle at 94 °C for 3 min, 35 cycles at 94 °C for 45 s, 55 °C for 60 s, 72 °C for 60 s, and a final cycle at 72 °C for 10 min. Before PCR-RFLP analysis, excess dNTPs and primers were removed from PCR products using a PCR purification kit (Solgent, Daejeon, Korea). Appropriate amounts of amplicon were digested at 37 °C for 1 h with 0.5 μl of *Hinf* I, *Hae* III, and *Hpa* I endonucleases (TaKaRa Shuzo Co.). DNA fragments were resolved by size on either a 5% polyacrylamide or 1.5% agarose gels, according to general methods (Sambrook and Russel, 2001). One kilobase or 100 bp ladders were used as markers for verifying DNA fragment sizes. To identify isolated yeasts, the nucleotide sequences of the ITS I-5.8S rDNA-ITS II region were compared with those available in the GenBank database using the BLAST feature of the National Center for Biotechnology Information database (Altschul et al., 1997; http://ncbi.nlm.nih.gov/BLAST).

2.2.3. Environmental tolerance analysis

Ethanol and glucose tolerances were established for the primary selection of isolated yeast that were likely to survive in the early and intermediate phases of wine fermentation. Each cell type was cultured in YPD broth consisting of 8% ethanol or 20% glucose at 30 °C for 48 h. Cell growth for each strain was measured using OD values at 600 nm. Strains that showed relatively high OD values in both analyses among similar species were selected. Only non-*Saccharomyces* yeasts were subjected to environmental tolerance analysis.

2.2.4. Sniffing test

For the final selection of persimmon wine and apple cider starters, a sniffing test was performed (IRB approval number: 2017-0125). The initial 5×10^6 CFU of 52 selected non-*Saccharomyces* yeast pellets were inoculated into 5 mL sterilized persimmon and apple juices and cultured at 20 °C for 14 days without shaking. Comprehensive evaluation of the odor intensity and pleasantness of the resulting wine was performed by a trained panel composed of five women and five men who were regarded as being sensitive at distinguishing odor. Each individual panelist evaluated the wine separately and then reached a consensus during a final group discussion. The final sniffing test scores ranged from + + + + + (very excellent), + + + (fair), to + (very poor).

2.2.5. Wine fermentation

To confirm the effect of non-*Saccharomyces* yeasts on wine quality, persimmons and 'fuji' apples that were cultivated in Cheong-Do and Cheong-Song, Korea, respectively, were used for wine fermentation. Persimmon and apple were washed, extracted, and adjusted to 24°Brix by adding the sugar prior to inoculation with yeasts. Each juice was fermented by a single-culture of non-*Saccharomyces* yeasts (*W. anomalus* SJ20, *H. uvarum* SJ69, *P. kluyveri* CD34, *S. bacillaris* CD80, and *M. carribica* YP1 for persimmon wine; *W. anomalus* CS7-16, and *S. bacillaris* CD80 for apple cider) and a mixed-culture of non-*Saccharomyces* yeasts and *S. cerevisiae* W-3 at a 9:1 (*v*/v) ratio at 20 °C until alcohol fermentation was completed. Control wine was fermented by single-culture of *S. cerevisiae* W-3. Potassium metabisulfite ($K_2S_2O_5$) was added at 200 mg/L to protect the wine from bacterial spoilage.

2.2.6. Analysis of wine characteristics

All the samples were centrifuged (3578 ×*g*, 10 min) to analyze the wine characteristics. Soluble solid was analyzed using a refractometer according to AOAC guidelines (Caputi Jr, 1995). The pH was measured, and the total acid content was determined by titration of the filtrates with 0.1 N NaOH (expressed as % acetic acid and malic acid for persimmon wine and apple cider, respectively). Alcohol content was measured using a hydrometer based on the specific gravity of wine distillates (expressed as % [v/v]) at 15 °C (Caputi Jr, 1995). Organic acid content was assayed by HPLC with a Shodex RSpak KC-811 column (diam. 8 × 300 mm, Showa Denko KK, Kawasaki, Japan). The column chromatography conditions were as follows: flow rate, 1 mL/min; temperature, 40 °C; and mobile phase, 0.1% phosphoric acid. Organic acids were detected using a refractive index detector (Hong and Park, 2013). The volatile ester compounds were determined using a gas chromatography mass spectrometer (7890A GC–MS; Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) followed as previously described (Lee et al., 2016b). The separation was performed with 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 of 1 mL/min. 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. Volatile ester compounds were collected using a solid-phase microextraction (SPME) fiber (50/30 μm DVB/CAR/PDMS; Supelco, Bellefonte, PA, USA). The extraction of volatile ester compounds from the wine was performed in headspace (HS) mode with magnetic stirring. Five milliliters of the sample was placed in an HS vial (20 mm, PTFE/silicon septum, magnetic cap), and 1.25 g of NaCl was added to increase volatile ester compound concentration in the HS. Prior to extraction, the sample was shaken in a water bath at 35 °C for 20 min to achieve equilibrium. Then, the SPME fiber was spiked into the vial and exposed at 35 °C for 40 min. The chemical standards were purchased from Chem Service Inc. (PA, USA). Volatile ester compound identification was based on a comparison of their GC retention times and mass spectrometry with spectral data from the Wiley9Nist 0.8 library (Wiley9ist 0.8 library, mass spectral search program, version 5.0, USA) (Torrens et al., 2004). The amount of each compound was calculated using the peak area based on the chemical standards.

2.2.7. Sensory evaluation

Sensory evaluation was carried out according to the five-point hedonic scale (Lee et al., 2016b). The panel was composed of 20 judges with sensitive taste discrimination from the Department of Food Science and Technology, Kyungpook National University, Korea. Sensory scores were 5 (excellent), 3 (fair), and 1 (very poor). All data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 12.0 for Windows) to obtain statistical information. ANOVA was conducted to compare the physicochemical characteristics and sensory properties of each sample and to test for significance between the average values of each measurement. Significance was determined to be $p < 0.05$ using the Duncan's multiple range test (Kim et al., 2008).

2.3. Optimization of air-blast dried non-Saccharomyces yeast starters

2.3.1. Excipient and protectant conditions

Lactomil (composed of 89% lactose and 11% maltodextrin; Seo Kang Dairy & Food Co., Ltd., Sacheon, Korea) and 10% skim milk were selected as a basic excipient and protectant respectively, in accordance with a previous study (Lee et al., 2016a). Six types of 10% sugar solutions (fructose, glucose, maltose, raffinose, sucrose, and trehalose) and two types of antioxidants (1, 3, and 5 mM of L-ascorbic acid and glutathione) were used as an extra protectant to examine starter survival rate immediately after air-blast drying and to assess any changes in starter viability during storage. Protective solutions consisting of skim milk and sugar were sterilized at 121 °C for 15 min, and antioxidant solutions were filter-sterilized. All the solutions were prepared and mixed aseptically just before the experiments.

2.3.2. Air-blast drying process

Each yeast strain was cultured in 100 mL YPD broth and incubated at 30 °C for 16 h. After culturing (approx. 1–2 × 10^8 CFU/mL), yeast cells were harvested by centrifugation (3578 ×*g* for 10 min) and rinsed twice in a 0.85% NaCl solution. The pellet was mixed with 7 g of lactomil, as well as 1 mL solutions of protective agents containing skim milk, sugar, and/or antioxidant. The mixed yeast cell pellets were dried using a Clear Air Oven (HB-509C, HanBaek, Bucheon, Korea) at 37 °C until the moisture content of the dried yeast starter had reached 10 ± 2%. After air-blast drying, the samples were immediately analyzed to determine the suitable moisture content and survival rate. These were then stored at 4 °C for two months, and the survival rate was determined again (Lee et al., 2016a).

2.3.3. Rehydration solution conditions

Air-blast dried samples were recovered to their original volume with four different rehydration solutions (sterilized distilled water, 1× PBS buffer, 0.85% NaCl, and 1% peptone water) by rehydration at room temperature for 1 h. The survival rate of each strain and treatment were investigated by serial dilution.

2.3.4. Measurement of survival rate and moisture content

Air-blast dried cells were recovered to their original volume with rehydration solutions. Then, the serially diluted samples were spread on YPD agar plates and incubated at 30 °C for 24 h. Any white colonies that formed on the YPD agar were counted. The survival rates of each sample were calculated as (%) survival = $(N/N_0) \times 100$, where N and N_0 represent the viable cell count number following or prior to air-blast drying (CFU/mL), respectively (Lee et al., 2016a). The moisture content of the air-blast dried yeast starters was estimated by measuring the weight loss after 10 h at 105 °C to determine the appropriate powder form (AOAC, 1990).

2.4. Morphology of air-blast dried yeast cells

The morphologies of air-blast dried non-*Saccharomyces* yeast cells were observed by SEM, as described by Hongpattarakere et al. (2013). The air-blast dried sample was affixed to "stubs" using double-sided metallic adhesive tape and then coated with gold using a Hitachi E-1030 ion sputter coater (Hitachi, Tokyo, Japan). The morphology of the sample was observed under a SU8220 scanning electron microscope (Hitachi, Tokyo, Japan) that was operated at an accelerating voltage of 5 kV. Images were obtained at 4000 × magnification. Scale bars were 10 μm as shown in each image.

2.5. Measurement of fermentation rate

The fermentation rates of yeast cells stored for 2 months after air-blast drying were analyzed, with cultured yeast cells used as the control. Each air-blast dried yeast cell was restored to its original volume with optimal rehydration solution for 1 h and 5% (v/v) of the cell suspension including a control was inoculated and incubated in 100 mL YPD broth containing 20% glucose (yeast extract 10 g/L, peptone 20 g/L, and glucose 200 g/L) at 30 °C with shaking (150 rpm) to measure the glucose consumption rate. A water trap apparatus containing concentrated H_2SO_4 was attached to the top of each flask to collect water evaporating during fermentation. The amount of CO_2 produced was directly measured as the decrease in the weight of the whole flask. The fermentation ratio was expressed as the percentage of the amount of CO_2 produced per the theoretical CO_2 production from the glucose owing to ethanol fermentation (Jung and Park, 2005).

2.6. Statistical analysis

All experiments were performed in triplicate and results analyzed using the Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows (IBM, Armonk, NY, USA) to establish statistical differences. Significance was determined at a threshold of $p < 0.05$ and $p < 0.01$ using one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test (Kim et al., 2008).

3. Results and discussion

3.1. Isolation of indigenous non-Saccharomyces yeasts

A total of 512 individual colonies were isolated from apple, aronia, Muscat Bailey A (MBA) grapes, and persimmon to develop indigenous non-*Saccharomyces* yeast starters for improving the quality of Korean persimmon wine and apple cider (Table 1). The ITS I-5.8S-ITS II region of each isolate was amplified, and six clearly different patterns were identified by PCR-restriction fragment length polymorphism (RFLP) analysis using three different endonucleases (*Hinf* I, *Hae* III, and *Hpa* I). *S. cerevisiae*, *W. anomalus*, and *H. uvarum* predominantly inhabited apple, aronia, and MBA grape cultivar samples, respectively, with *S. bacillaris*, *P. kluyveri*, and *M. caribbica* also isolated. In comparison to

Table 2
Primary selection of isolated non-*Saccharomyces* yeasts, depending on environmental tolerances.

	Isolate	Species	Cell growth (OD 600 nm)		Origin
			20% glucose	8% EtOH	
1	SJ1	*W. anomalus*	23.96 ± 0.56	4.98 ± 0.05	Persimmon
2	SJ2	*W. anomalus*	21.96 ± 0.16	7.23 ± 0.04	Persimmon
3	SJ7	*W. anomalus*	22.22 ± 0.14	6.06 ± 0.06	Persimmon
4	SJ11	*W. anomalus*	23.03 ± 0.48	6.17 ± 0.02	Persimmon
5	SJ15	*W. anomalus*	21.61 ± 0.18	6.47 ± 0.08	Persimmon
6	SJ16	*W. anomalus*	22.13 ± 0.98	4.39 ± 0.01	Persimmon
7	SJ18	*W. anomalus*	22.07 ± 0.30	6.12 ± 0.07	Persimmon
8	SJ19	*W. anomalus*	22.95 ± 0.23	6.39 ± 0.06	Persimmon
9	SJ20	*W. anomalus*	21.59 ± 0.06	6.06 ± 0.05	Persimmon
10	SJ24	*W. anomalus*	13.75 ± 0.06	7.44 ± 0.02	Persimmon
11	SJ37	*W. anomalus*	22.55 ± 0.19	4.29 ± 0.10	Persimmon
12	SJ47	*W. anomalus*	10.79 ± 0.04	6.67 ± 0.02	Persimmon
13	SJ53	*H. uvarum*	12.04 ± 0.04	0.88 ± 0.01	Persimmon
14	SJ61	*H. uvarum*	6.88 ± 0.03	1.94 ± 0.01	Persimmon
15	SJ68	*H. uvarum*	9.85 ± 0.03	1.05 ± 0.01	Persimmon
16	SJ69	*H. uvarum*	7.75 ± 0.12	1.04 ± 0.02	Persimmon
17	SJ77	*H. uvarum*	9.63 ± 0.02	1.21 ± 0.01	Persimmon
18	SJ81	*H. uvarum*	9.05 ± 0.05	1.16 ± 0.01	Persimmon
19	SJ88	*H. uvarum*	8.91 ± 0.21	1.20 ± 0.01	Persimmon
20	CD1	*P. kluyveri*	7.45 ± 0.06	1.43 ± 0.06	Persimmon
21	CD2	*P. kluyveri*	10.24 ± 0.15	1.20 ± 0.01	Persimmon
22	CD3	*P. kluyveri*	10.66 ± 0.11	0.36 ± 0.01	Persimmon
23	CD8	*H. uvarum*	12.59 ± 0.29	0.98 ± 0.01	Persimmon
24	CD14	*H. uvarum*	13.00 ± 0.22	1.04 ± 0.01	Persimmon
25	CD23	*P. kluyveri*	12.68 ± 0.25	0.41 ± 0.02	Persimmon
26	CD25	*P. kluyveri*	8.96 ± 0.09	1.01 ± 0.01	Persimmon
27	CD34	*P. kluyveri*	7.11 ± 0.07	1.04 ± 0.02	Persimmon
28	CD35	*H. uvarum*	7.11 ± 0.06	0.98 ± 0.02	Persimmon
29	CD39	*H. uvarum*	9.48 ± 0.04	1.06 ± 0.02	Persimmon
30	CD72	*P. kluyveri*	6.39 ± 0.12	0.70 ± 0.01	Persimmon
31	CD75	*P. kluyveri*	7.71 ± 0.02	1.03 ± 0.01	Persimmon
32	CD76	*P. kluyveri*	7.64 ± 0.10	0.87 ± 0.03	Persimmon
33	CD77	*H. uvarum*	9.77 ± 0.12	1.02 ± 0.02	Persimmon
34	CD78	*H. uvarum*	7.32 ± 0.03	1.22 ± 0.01	Persimmon
35	CD80	*S. bacillaris*	9.84 ± 0.07	1.08 ± 0.01	Persimmon
36	CD81	*S. bacillaris*	9.81 ± 0.10	1.17 ± 0.01	Persimmon
37	CD82	*H. uvarum*	9.81 ± 0.07	1.42 ± 0.02	Persimmon
38	CD83	*P. kluyveri*	8.35 ± 0.17	1.21 ± 0.01	Persimmon
39	GJ14	*W. anomalus*	27.40 ± 0.12	4.55 ± 0.01	Aronia
40	GJ16	*W. anomalus*	24.34 ± 0.02	4.98 ± 0.02	Aronia
41	YP1	*M. caribbica*	16.58 ± 0.06	1.51 ± 0.01	Aronia
42	YP5	*W. anomalus*	27.40 ± 0.24	5.71 ± 0.41	Aronia
43	YP9	*W. anomalus*	25.10 ± 0.03	6.16 ± 0.21	Aronia
44	YP24	*W. anomalus*	26.98 ± 0.21	5.33 ± 0.14	Aronia
45	YP30	*W. anomalus*	27.44 ± 0.14	5.89 ± 0.21	Aronia
46	YP37	*W. anomalus*	29.62 ± 0.21	5.25 ± 0.15	Aronia
47	YP38	*W. anomalus*	29.26 ± 0.41	5.48 ± 0.04	Aronia
48	YP39	*W. anomalus*	29.75 ± 0.12	4.98 ± 0.13	Aronia
49	YP40	*W. anomalus*	28.34 ± 0.21	5.72 ± 0.19	Aronia
50	DY32	*M. caribbica*	21.33 ± 0.03	1.57 ± 0.04	Aronia
51	CS7–16	*W. anomalus*	30.48 ± 0.21	4.33 ± 0.12	Apple
52	CS13–15	*W. anomalus*	31.24 ± 0.32	5.60 ± 0.21	Apple

Table 1
Sizes of the ITS-5.8S-ITS II gene amplicon and restriction fragments of isolates and the number of strains depending on the origin.

Strains	Amplicon size (bp)	Size of restriction fragments (bp)			Number of strains depending on the origin				Total
		Hinf I	*Hae* III	*Hpa* I	Apple	Aronia	MBA grapes	Persimmon	
S. cerevisiae	810	100 + 360 + 340	110 + 170 + 220 + 310	230 + 580	81	2		7	90
H. uvarum	720	60 + 140 + 190 + 330	720	720	1	1	34	101	137
S. bacillaris	720	225 + 225	450	450			2	2	4
W. anomalus	600	410 + 190	600	390 + 300	3	195		71	269
P. kluyveri	440	200 + 240	70 + 370	440				10	10
M. caribbica	600	310 + 380	80 + 100 + 390	600		2			2
Total					85	200	36	191	512

previous findings, Valles et al. (2007) reported that the diversity of yeast is influenced by several factors, including geographic region, climate conditions, and varieties. Various yeast species, such as *Hansenia* sp., *Metschnikowia pulcherrima*, *P. guillermondii*, and *Saccharomyces* sp., have been identified from apple cider during spontaneous fermentation. In another study by Hidalgo et al. (2012), eight species of yeast, including *Saccharomyces* and non-*Saccharomyces* yeasts, were isolated and identified from the fermentation of persimmon. Among the yeasts isolated in the present study, 422 non-*Saccharomyces* yeasts, considered likely to produce volatile aromatic compounds in persimmon wine and apple cider, were used for subsequent experiments.

3.2. Selection of persimmon wine and apple cider yeast starters using environmental tolerance and sniffing test analyses

We measured the environmental tolerances of isolated yeasts cultivated for 48 h on YPD broth modified to be similar to the early and intermediate stages of wine fermentation. We selected 24 *W. anomalus* and 14 *H. uvarum* strains that showed relatively high cell growth among the same species (Table 2). Each of the *S. bacillaris*, *P. kluyveri*, and *M. caribbica* isolates were also selected because of the low numbers of isolated candidates. The 52 selected non-*Saccharomyces* yeasts were then evaluated for their aroma producing activity using a sniffing test. The cell growth of yeast during fermentation is affected by several factors such as temperature, pH, ethanol, and glucose concentration (Charoenchai et al., 1998; Gao and Fleet, 1988; Pina et al., 2004). In particular, several non-*Saccharomyces* yeasts are unable to survive in high concentrations of ethanol produced during fermentation, with a maximum tolerance of 6% v v^{-1} often being cited (Fleet, 1990; Gil et al., 1996; Pina et al., 2004). These yeasts proliferate slowly because of an osmotic effect created by high glucose concentrations (Leslie et al., 1995), indicating the importance of selecting non-*Saccharomyces* strains with high tolerance to various environmental stresses.

For the sniffing test, 52 non-*Saccharomyces* yeasts were inoculated into 5 mL aliquots of sterilized persimmon and apple juice and then fermented at 20 °C for two weeks. Final selections of yeast starters potentially producing high levels of aromatic compounds for persimmon wine and apple cider were conducted by integrating the discussion of 10 trained panelists (Table 3). Among the non-*Saccharomyces* yeasts that were inoculated into the persimmon juice, *W. anomalus* SJ20, *H. uvarum* SJ69, *P. kluyveri* CD34, *S. bacillaris* CD80, and *M. caribbica* YP1 received higher scores and were selected as persimmon wine yeast starters. In addition, *W. anomalus* CS7-16 and *S. bacillaris* CD80 were selected as apple cider yeast starters with relatively high scores in the sniffing test. Several enzymes produced by non-*Saccharomyces* yeasts, such as protease, β-glucosidase, esterase, pectinase, and lipase, can affect the quality of wine by improving juice clarification and wine filtration (pectinase and protease) and by releasing aromatic compounds (esterase and β-glucosidase) and free fatty acids into the juice or wine (lipase) (Esteve-Zarzoso et al., 1998; Mateo and Maicas, 2016). Moreover, *Candida* sp., *Pichia* sp., and *Hanseniaspora* sp. isolated from the various fruits in this study have also been reported to generate multiple volatile aromatic compounds in wine. This is also expected to apply to persimmon wine and apple cider (Englezos et al., 2016; Swangkeaw et al., 2011; Ye et al., 2014).

Table 3
Sniffing test scores of primary selected yeasts for final selection.

Isolate	Aroma intensity	
	Persimmon juice	Apple juice
W. anomalus		
SJ1	+	+ +
SJ2	+ + +	+
SJ7	+	+
SJ11	+	+
SJ15	+	+
SJ16	+	+ +
SJ18	+ + +	+ +
SJ19	+ + +	+ +
SJ20*	+ + + + +	+ +
SJ24	+	+
SJ37	+	+
SJ47	+	+
GJ14	+ + +	+ +
GJ16	+ + +	+ +
YP5	+ + +	+
YP9	+	+
YP24	+	+
YP30	+	+
YP37	+	+
YP38	+	+ +
YP39	+	+
YP40	+	+ +
CS7-16*	+ +	+ + + +
CS13-15	+ +	+ +
S. bacillaris		
CD80*	+ + + +	+ + +
CD81	+ +	+ +
H. uvarum		
SJ53	+ + +	+ +
SJ61	+ + +	+
SJ68	+ + +	+ +
SJ69*	+ + + +	+ +
SJ77	+	+ +
SJ81	+ +	+ +
SJ88	+ +	+ +
CD8	+ +	+
CD14	+	+ +
CD35	+	+
CD39	+	+ +
CD77	+	+ +
CD78	+	+
CD82	+ + +	+ +
P. kluyveri		
CD1	+ +	+
CD2	+	+ +
CD3	+	+ +
CD23	+ +	+
CD25	+ +	+ +
CD34*	+ + + +	+ +
CD72	+ +	+ +
CD75	+	+ +
CD76	+	+
CD83	+ +	+
M. caribbica		
DY32	+	+
YP1*	+ + + +	+ +

*Bold isolates indicate final non-*Saccharomyces* yeast strains selected by a 10-person panel that scored odor and taste after fermentation, ranging from + + + + + (very excellent), + + + (fair), to + (unpleasant).

3.3. Effect of non-Saccharomyces yeasts on persimmon wine and apple cider characteristics

The fermentation characteristics of persimmon wines and apple ciders single-fermented with non-*Saccharomyces* yeasts or mixed-fermented with both non-*Saccharomyces* yeasts selected by the previous selection procedures and *S. cerevisiae* W-3 are shown in Tables 4 and 5 to determine the effect of those strains on apple cider and persimmon wine fermentation. Alcohol fermentation of persimmon wine and apple cider fermented by single fermentation with non-*Saccharomyces* yeasts was completed within 13–19 days, and alcohol fermentation of persimmon wine and apple cider fermented by mixed fermentation and single fermentation with *S. cerevisiae* W-3 (control) was completed within 9 days. In the results of persimmon wine, alcohol contents of mix-fermented persimmon wines were similar to that of the control, whereas that of persimmon wines fermented by single fermentation except for *M. caribbica* YP1 was lower than that of the control. Moreover, the soluble solid contents of persimmon wines fermented by single fermentation were slightly higher than those of the control. The pH and

Table 4

Physicochemical properties and sensory scores of the persimmon wines fermented with single or co-culture of non-*Saccharomyces* yeasts such as *W. anomalus* SJ20 (SJ20), *H. uvarum* SJ69 (SJ69), *P. kluyveri* CD34 (CD34), *S. bacillaris* CD80 (CD80), and *M. caribbica* YP1 (YP1) and *S. cerevisiae* W-3.

Property	Strains										
	W-3	SJ20	SJ69	CD34	CD80	YP1	W3 + SJ20	W3 + SJ69	W3 + CD34	W3 + CD80	W3 + YP1
Fermentation time (h)	216	360	312	312	408	360	216	216	216	216	216
Alcohol (%, *v*/v)	12.9 ± 0.2^{A}	12.1 ± 0.1^{C}	11.8 ± 0.1^{CD}	11.6 ± 0.2^{DE}	11.4 ± 0.1^{DE}	12.9 ± 0.3^{A}	12.4 ± 0.2^{B}	12.5 ± 0.2^{AB}	12.7 ± 0.1^{AB}	12.5 ± 0.2^{AB}	12.6 ± 0.2^{AB}
Soluble solid (°Brix)	7.3 ± 0.1^{BC}	7.0 ± 0.0^{D}	8.0 ± 0.1^{A}	7.6 ± 0.1^{B}	8.0 ± 0.2^{A}	7.4 ± 0.2^{BC}	7.4 ± 0.1^{BC}	7.2 ± 0.1^{CD}	7.6 ± 0.2^{B}	7.6 ± 0.1^{B}	7.6 ± 0.1^{B}
pH	3.99 ± 0.02^{D}	4.10 ± 0.02^{B}	4.20 ± 0.01^{A}	4.00 ± 0.02^{D}	4.04 ± 0.03^{D}	4.03 ± 0.02^{CD}	4.10 ± 0.01^{B}	4.11 ± 0.02^{B}	4.06 ± 0.02^{BC}	4.09 ± 0.03^{B}	4.09 ± 0.02^{B}
Total acidity (%)	0.54 ± 0.04^{B}	0.56 ± 0.03^{B}	0.74 ± 0.05^{A}	0.58 ± 0.03^{B}	0.71 ± 0.04^{A}	0.51 ± 0.04^{B}	0.48 ± 0.05^{B}	0.50 ± 0.03^{B}	0.54 ± 0.03^{B}	0.47 ± 0.04^{B}	0.49 ± 0.05^{B}
Organic acid (mg/mL)											
Citric acid	1.04 ± 0.12^{A}	0.76 ± 0.00^{C}	0.98 ± 0.03^{A}	0.84 ± 0.02^{B}	0.73 ± 0.02^{C}	0.88 ± 0.02^{B}	1.13 ± 0.10^{A}	1.15 ± 0.00^{A}	1.12 ± 0.10^{A}	1.13 ± 0.12^{A}	1.12 ± 0.11^{A}
Malic acid	3.88 ± 0.23^{A}	3.85 ± 0.20^{A}	2.20 ± 0.07^{C}	4.02 ± 0.30^{A}	2.97 ± 0.05^{B}	3.84 ± 0.20^{A}	3.31 ± 0.20^{A}	3.21 ± 0.18^{A}	3.60 ± 0.20^{A}	2.76 ± 0.04^{B}	3.26 ± 0.17^{A}
Acetic acid	0.28 ± 0.02^{E}	0.32 ± 0.00^{E}	3.30 ± 0.18^{A}	0.48 ± 0.01^{C}	0.90 ± 0.01^{B}	0.13 ± 0.00^{F}	0.08 ± 0.02^{G}	0.15 ± 0.00^{F}	0.29 ± 0.00^{E}	0.37 ± 0.00^{D}	0.26 ± 0.03^{E}
Volatile ester compounds (μg/mL)											
Methyl acetate	6.1 ± 0.5^{D}	58.2 ± 5.3^{A}	28.9 ± 3.1^{B}	11.8 ± 2.2^{D}	6.3 ± 0.7^{D}	8.7 ± 0.8^{D}	8.5 ± 0.7^{D}	8.8 ± 0.8^{D}	7.7 ± 0.6^{D}	9.5 ± 0.8^{D}	21.6 ± 1.8^{C}
Ethyl acetate	283.4 ± 31.5^{C}	1981.4 ± 216.4^{A}	1974.1 ± 187.3^{A}	267.6 ± 23.6^{C}	371.6 ± 41.2^{C}	280.8 ± 25.9^{C}	381.7 ± 35.3^{C}	302.7 ± 28.6^{C}	339.4 ± 31.6^{C}	318.4 ± 30.5^{C}	1023.3 ± 96.9^{B}
Ethyl hexanoate	297.0 ± 25.5^{B}	303.9 ± 33.0^{B}	37.4 ± 3.4^{D}	426.8 ± 41.5^{A}	16.7 ± 2.1^{D}	401.0 ± 42.2^{A}	202.3 ± 21.4^{C}	202.4 ± 19.8^{C}	220.8 ± 21.1^{C}	177.4 ± 15.6^{C}	201.2 ± 19.7^{C}
Ethyl heptanoate	5.1 ± 0.4^{BC}	5.9 ± 0.5^{ABC}	4.6 ± 0.4^{C}	6.4 ± 0.7^{AB}	5.9 ± 0.5^{ABC}	5.9 ± 0.7^{ABC}	4.9 ± 0.6^{C}	4.8 ± 0.5^{C}	5.3 ± 0.6^{BC}	6.8 ± 0.4^{A}	5.0 ± 0.4^{BC}
Ethyl octanoate	382.9 ± 41.1^{BC}	437.2 ± 46.3^{B}	48.7 ± 5.1^{D}	586.2 ± 60.2^{A}	24.1 ± 2.5^{D}	470.2 ± 43.5^{B}	432.2 ± 38.7^{B}	472.2 ± 45.5^{B}	483.1 ± 43.1^{B}	437.6 ± 44.7^{B}	335.2 ± 36.5^{C}
Methyl salicylate	62.9 ± 7.1^{A}	42.1 ± 5.3^{CD}	60.6 ± 5.8^{A}	59.2 ± 6.1^{AB}	49.3 ± 4.8^{ABCD}	56.6 ± 5.9^{ABC}	53.2 ± 5.1^{ABCD}	54.1 ± 5.3^{ABCD}	65.4 ± 8.3^{A}	39.6 ± 5.6^{D}	44.5 ± 5.0^{BCD}
2-Phenylethyl acetate	192.5 ± 20.5^{B}	90.7 ± 10.4^{C}	17.5 ± 2.8^{D}	170.9 ± 21.7^{B}	16.3 ± 1.3^{D}	271.0 ± 28.9^{A}	112.4 ± 13.4^{C}	96.5 ± 11.5^{C}	88.9 ± 9.8^{C}	77.5 ± 8.4^{C}	95.4 ± 9.9^{C}
Sensory score											
Color	3.80 ± 0.39^{A}	3.80 ± 0.48^{A}	3.35 ± 0.47^{A}	3.80 ± 0.51^{A}	3.70 ± 0.46^{A}	3.80 ± 0.51^{A}	3.65 ± 0.67^{A}	3.55 ± 0.48^{A}	3.55 ± 0.50^{A}	3.50 ± 0.45^{A}	3.10 ± 0.56^{A}
Flavor	3.00 ± 0.55^{A}	3.20 ± 0.66^{A}	3.35 ± 0.62^{A}	3.30 ± 0.52^{A}	3.00 ± 0.67^{A}	3.20 ± 0.66^{A}	3.50 ± 0.59^{A}	3.15 ± 0.50^{A}	3.10 ± 0.36^{A}	2.85 ± 0.44^{A}	3.65 ± 0.55^{A}
Taste	2.65 ± 0.59^{A}	2.90 ± 0.73^{A}	3.50 ± 0.58^{A}	2.65 ± 0.47^{A}	3.10 ± 0.73^{A}	2.60 ± 0.53^{A}	2.90 ± 0.67^{A}	2.90 ± 0.61^{A}	2.80 ± 0.66^{A}	2.55 ± 0.59^{A}	2.55 ± 0.53^{A}
Overall preference	3.00 ± 0.49^{A}	3.05 ± 0.62^{A}	3.35 ± 0.55^{A}	3.00 ± 0.46^{A}	3.35 ± 0.64^{A}	2.95 ± 0.64^{A}	3.30 ± 0.52^{A}	3.05 ± 0.55^{A}	2.75 ± 0.63^{A}	2.60 ± 0.53^{A}	2.85 ± 0.52^{A}

Different letters within the same horizontal line indicate significant difference ($p < 0.05$).

Table 5

Physicochemical properties and sensory scores of the apple ciders fermented with single or co-culture of non-*Saccharomyces* yeasts such as *W. anomalus* CS7-16 (CS7-16), and *S. bacillaris* CD80 (CD80) and *S. cerevisiae* W-3.

Property	Strains				
	W-3	CS7-16	CD80	W3 + CS7-16	W3 + CD80
Fermentation time (h)	216	360	456	216	216
Alcohol (%, v/v)	13.1 ± 0.2^{A}	11.2 ± 0.2^{B}	11.2 ± 0.2^{B}	13.1 ± 0.2^{A}	13.1 ± 0.1^{A}
Soluble solid (°Brix)	8.6 ± 0.2^{B}	9.8 ± 0.2^{A}	9.8 ± 0.1^{A}	8.4 ± 0.1^{B}	8.4 ± 0.1^{B}
pH	3.82 ± 0.02^{B}	3.98 ± 0.01^{A}	3.99 ± 0.02^{A}	3.87 ± 0.01^{B}	3.83 ± 0.02^{B}
Total acidity (%)	0.64 ± 0.03^{B}	0.53 ± 0.03^{C}	0.50 ± 0.02^{C}	0.64 ± 0.04^{B}	0.72 ± 0.02^{A}
Organic acid (mg/mL)					
Citric acid	1.18 ± 0.12^{B}	1.20 ± 0.17^{B}	2.08 ± 0.26^{A}	1.23 ± 0.14^{B}	1.31 ± 0.16^{B}
Malic acid	5.74 ± 0.89^{B}	2.66 ± 0.45^{D}	3.97 ± 0.59^{C}	7.95 ± 0.86^{A}	6.63 ± 0.92^{AB}
Lactic acid	0.93 ± 0.06^{AB}	0.66 ± 0.03^{C}	0.98 ± 0.08^{A}	0.82 ± 0.04^{B}	0.80 ± 0.09^{B}
Volatile ester compounds (μg/mL)					
Ethyl acetate	283.7 ± 31.4^{AB}	1148.6 ± 126.8^{A}	270.9 ± 25.6^{B}	332.2 ± 35.5^{B}	233.5 ± 19.8^{B}
Isobutyl acetate	4.2 ± 0.5^{B}	3.9 ± 0.5^{B}	6.9 ± 0.8^{A}	6.1 ± 0.6^{A}	3.1 ± 0.4^{B}
Ethyl butanoate	18.4 ± 2.2^{B}	28.7 ± 2.7^{A}	8.1 ± 0.9^{C}	10.3 ± 1.2^{C}	18.1 ± 1.6^{B}
Isoamyl acetate	351.7 ± 33.6^{A}	24.6 ± 2.8^{D}	19.8 ± 2.0^{D}	251.3 ± 21.4^{B}	85.8 ± 9.6^{C}
Ethyl hexanoate	328.9 ± 29.5^{A}	6.6 ± 0.8^{C}	19.0 ± 2.2^{C}	222.3 ± 20.5^{B}	315.9 ± 32.8^{A}
Hexyl acetate	26.7 ± 2.4^{A}	0.6 ± 0.1^{D}	1.2 ± 0.2^{D}	9.8 ± 1.0^{B}	5.1 ± 0.4^{C}
Ethyl octanoate	1331.3 ± 150.5^{B}	97.9 ± 9.2^{C}	16.9 ± 1.7^{D}	1259.6 ± 114.5^{B}	1575.9 ± 131.4^{A}
Methyl salicylate	9.3 ± 0.9^{C}	12.2 ± 1.3^{AB}	13.4 ± 1.0^{A}	8.5 ± 0.7^{C}	10.5 ± 0.9^{BC}
2-Phenylethyl acetate	151.2 ± 12.5^{A}	15.1 ± 1.8^{D}	18.1 ± 2.1^{D}	28.6 ± 2.5^{C}	58.6 ± 6.1^{B}
Sensory score					
Color	3.60 ± 0.44^{A}	3.35 ± 0.68^{A}	3.70 ± 0.40^{A}	3.35 ± 0.34^{A}	3.85 ± 0.50^{A}
Flavor	3.35 ± 0.44^{A}	3.25 ± 0.38^{A}	2.35 ± 0.47^{B}	3.30 ± 0.54^{A}	2.35 ± 0.52^{B}
Taste	3.75 ± 0.50^{A}	3.30 ± 0.71^{A}	2.50 ± 0.44^{B}	2.50 ± 0.50^{B}	2.55 ± 0.44^{B}
Overall preference	3.80 ± 0.46^{A}	3.65 ± 0.51^{A}	2.90 ± 0.55^{B}	3.75 ± 0.39^{A}	2.90 ± 0.50^{B}

Different letters within the same horizontal line indicate significant difference ($p < 0.05$).

total acidity of all the persimmon wines were detected as 3.99–4.20 and 0.47–0.74%, respectively. Citric acid contents of persimmon wines fermented by mixed fermentation were higher than those of the control, whereas those of persimmon wines fermented by single fermentation were lower than those of the control. Malic acid contents of persimmon wines fermented by both single and mixed fermentation with *H. uvarum* SJ69 and *S. bacillaris* CD80 were statistically lower than those of the control. The highest acetic acid content was detected in persimmon wine fermented by single fermentation with *H. uvaum* SJ69. The differences in volatile ester compounds were larger in persimmon wines fermented by single fermentation than in persimmon wines fermented by mixed fermentation. Methyl acetate and ethyl acetate contents of persimmon wine fermented by single fermentation with *W. anomalus* SJ20 and *H. uvarum* SJ69, and mixed fermentation with *M. caribbica* YP1 were significantly higher than those of the control and were increased for the other wines in most cases. In addition, persimmon wines fermented by single fermentation with *W. anomalus* SJ20, *P. kluyveri* CD34, and *M. caribbica* YP1 had higher contents of ethyl hexanoate and ethyl octanoate than the control, whereas persimmon wines fermented by single fermentation with *H. uvarum* SJ69 and *S. bacillaris* CD80 had lower contents of ethyl hexanoate and ethyl octanoate than the control. For persimmon wines fermented by mixed fermentation, ethyl hexanoate contents were decreased compared to the control, whereas ethyl octanoate contents were increased compared to the control except for *M. caribbica* YP1. Ethyl heptanoate and methyl salicylate contents did not show significant differences among all the persimmon wines. 2-phenyletheyl acetate content of persimmon wine fermented by single fermentation with *M. caribbica* YP1 was higher than that of the control, whereas those of other wines were lower than that of the control. For the results of sensory evaluation, all the persimmon wines fermented by single and mixed fermentation, except for *S. bacillaris* CD80, obtained higher flavor scores than the control wine. Similarly, taste and overall preference were high for most wines fermented by single and mixed fermentation compared with control wine. Some of the wines that obtained lower scores in the sensory evaluation showed no statistically significant differences compared to the control wine.

For the results of apple ciders, alcohol contents of apple cider fermented by single fermentation with *W. anomalus* CS7–16 and *S. bacillaris* CD80 were higher than those of the control, but the soluble solid contents were lower than those of the control. The pH of apple ciders fermented by single and mixed fermentation was higher than that of the control, whereas the total acidity of apple ciders fermented by single fermentation was lower than that of the control. Citric acid content of apple cider fermented by single fermentation with *S. bacillaris* CD80 was higher than that of the other ciders. Malic acid contents of apple ciders fermented by single fermentation were lower than those of the control; moreover, the lowest lactic acid content was detected in apple cider fermented by single fermentation with *W. anomalus* CS7-16. Ethyl acetate contents of apple ciders fermented by single and mixed fermentation with *W. anomalus* CS7-16 were higher than that of the control, whereas the isobutyl acetate contents of all ciders were not significantly different. Apple cider fermented by single fermentation with *W. anomalus* CS7-16 had a higher ethyl butanoate content than the control, whereas the other ciders had a lower content than the control or no significant difference. Isoamyl acetate, ethyl hexanoate, hexyl acetate, ethyl octanoate, and 2-phenylethyl acetate contents of apple ciders fermented by single fermentation were considerably lower than those of the control, whereas those of apple ciders fermented by mixed fermentation were also lower than those of the control but higher than those of apple ciders fermented by single fermentation (except for ethyl octanoate content in apple cider fermented by mixed fermentation with *S. bacillaris* CD80). Methyl salicylate contents of apple ciders fermented by single fermentation were higher than those of the control, whereas those of apple ciders fermented by mixed fermentation were similar to those of the control. Sensory evaluation analysis showed that apple ciders fermented by single and mixed fermentation with *W. anomalus* CS7-16 obtained similar flavor and overall preference scores compared to those of the control, whereas apple ciders fermented by single and mixed fermentation with *S. bacillaris* CD80 had the worse flavor and overall preference scores compared to those of the control because of the reduced volatile ester compounds during alcohol fermentation.

Several studies reported that non-*Saccharomyces* yeasts showed low ethanol productivity and late fermentation time during wine fermentation compared to *S. cerevisiae* (Ciani and Picciotti, 1995; Toro and Vazquez, 2002). Similar to those results, persimmon wines and apple ciders fermented by single fermentation with non-*Saccharomyces* yeasts in the present study showed lower ethanol contents and delayed fermentation time compared to those of the control. Compared to the result of mixed-fermentation, persimmon wines and apple ciders fermented by single fermentation helped to clearly determine the differences in the secondary metabolites of the wines and ciders derived from the different yeast species. Interestingly, ethyl acetate contents were dramatically increased when persimmon wines were fermented by single fermentation with *W. anomalus* SJ20 and *H. uvarum* SJ69 and when apple cider was fermented by single fermentation with *W. anomalus* CS7-16. Although ethyl acetate is one of the major aromatic compounds produced by non-*Saccharomyces* yeasts, some studies reported that excessively high concentration of ethyl acetate does not enhance wine aroma (Lilly et al., 2000; Rojas et al., 2001). Thus, appropriately controlling the mixed fermentation with non-*Saccharomyces* yeasts and *S. cerevisiae* for a suitable ethyl acetate content might contribute to the improvement of the quality of persimmon wine and apple cider. Several non-*Saccharomyces* yeasts such as *W. anomalus* SJ20, *H. uvarum* SJ69, and *W. anomalus* CS7-16 showed the best potential as persimmon wine and apple cider starters according to the result of volatile ester compounds and sensory evaluation; moreover, other strains also had a fine potential as wine starters for making distinctive types of persimmon wine and apple cider because of the different compositions of the various volatile ester compounds compared to others.

3.4. Effect of various sugar additives as protectants and rehydration solutions on the survival rate of air-blast dried non-Saccharomyces yeast cells

To develop non-*Saccharomyces* yeast wine starters for persimmon wine and apple cider, six kinds of non-*Saccharomyces* yeasts selected by the previous two-step environmental tolerance and sniffing test screening procedures were air-blast dried at 37 °C until the moisture content of the cells reached 10 ± 2%. Protective agents, composed primarily of 10% skim milk and six kinds of sugar (fructose, glucose, maltose, raffinose, sucrose, and trehalose), were investigated with regards to their ability to improve the survival rate of air-blast dried non-*Saccharomyces* yeast cells (Fig. 1). According to a previous study (Lee et al., 2016a), 7 g of lactomil used as an excipient could lead to a stable powdered form in the final dried product. Rehydration conditions, an important factor in the recovery of yeast starters, were investigated using four kinds of rehydration solutions (sterilized distilled water, 1 × phosphate buffered saline [PBS] buffer, 0.85% NaCl, and 1% peptone water) at room temperature for 1 h (Fig. 1). The sample with no added sugar and rehydrated by sterilized distilled water was used as a control. Each strain exhibited different optimal protectant and rehydration solution conditions. For *W. anomalus* SJ20, all the sugars added to the samples, except for 10% raffinose, yielded considerably higher survival rates than the control. In particular, the addition of 10% trehalose and rehydration using 1% peptone water resulted in the highest survival rate of 103.76%. This was 82.21% higher than the control. For *H. uvarum* SJ69, the survival rates of most of the samples using fructose, maltose, sucrose, and trehalose as a protectant were increased compared to the control. In particular, the addition of 10% sucrose and rehydration using 0.85% NaCl resulted in the highest survival rate of 144.33%. This was 60.26% higher than that of the control. For *P. kluyveri* CD34, most samples did not show any variance in their survival rate. Of the samples examined, only two demonstrated an increased survival rate, with the addition of 10% fructose, and rehydration using 0.85% NaCl resulting in the highest survival rate of 182.10%. This was 105.49% higher than the control. For *S. bacillaris* CD80, the addition of 10% sucrose and rehydration using 0.85% NaCl resulted in the highest survival rate of 164.61%, which was 79.88% higher than the control. In contrast, the other samples did not show a significant increase in survival rate compared to the control. For *M. caribbica* YP1, several samples showed considerably higher survival rates than the control. Among these, the addition of 10% trehalose and rehydration using 1 × PBS buffer resulted in the highest survival rate of 160.69%. This was 105.49% higher than that of the control. For *W. anomalus* CS7-16, the addition of trehalose yielded the highest protective effect among sugar additives, and rehydration using 0.85% NaCl resulted in the highest survival rate of 152.46%, 112.18% higher than that of the control.

The selection of protectants is also very important for the viability of microorganism starters because intracellular accumulation of suitable solutes is associated with strain survival after drying. Use of an appropriate protectant contributes to the protection of the proteins and membranes of the microorganism (Champagne and Gardner, 2001; Champagne et al., 2012; Lee et al., 2016a). For example, a study by Leslie et al. (1995) found that the survival rates of freeze-dried *Escherichia coli* and *Bacillus thuringiensis* increased relative to controls when trehalose and sucrose were used as protectants. This was similar to the finding in our study in which trehalose and sucrose had the greatest effects on protecting survival rates in the majority of air-blast dried yeast cells. In addition, rehydration also represents an essential step in recovering dried microorganisms. If dried cells are exposed to inappropriate conditions, such as an unfavorable osmotic environment, it may be challenging for them to recover from the resulting injured state (De Valdez et al., 1985a; Lee et al., 2016b; Zhao and Zhang, 2005). Alternatively, Abadias et al. (2001) reported that the viability of freeze-dried *Candida sake* increases when rehydrated in various protective media such as 10% skim milk, 1% peptone water, and the same solution used as protectant. In turn, De Valdez et al. (1985b) showed that diluent medium (1.5% peptone, 1% tryptone, and 0.5% meat extract) afforded the best recovery for freeze-dried lactic acid bacteria.

Finally, Lee et al. (2016b) demonstrated that the survival rate of freeze-dried *Lactobacillus plantarum* JH287 increased when cells were rehydrated for 1 h compared to 0 h. Teixeira et al. (1995a) reported that slow rehydration (soaking) showed a higher survival rate than rapid rehydration (shaking) for *Lactobacillus bulgaricus* starter prepared by spray drying. Similarly, Poirier et al. (1999) revealed that slow rehydration allowed an increase in the viability of *S. cerevisiae* compared with rapid dehydration since slow rehydration could allow slow cell water uptake by an unstable cell membrane to preserve cell viability. A study by Jenkins et al. (2011) also reported that the viability of active dry brewing yeast cells was increased by rehydration for 1 h by recovering their capacity to replicate when fully rehydrated.

3.5. Effect of antioxidant additives on the storability of air-blast dried non-Saccharomyces yeast starter products

In the microorganism starter industry, the storage period of a product is extremely important. Previously, several studies have reported that cell membrane lipid oxidation is related to the viability of dried cells during storage (Teixeira et al., 1996; Yao et al., 2008). This work has also determined that some antioxidants such as L-ascorbic acid and glutathione exert a beneficial effect on the storage of starter products (Dave and Shah, 1997; Teixeira et al., 1995b; Zhang et al., 2010). In the present study, changes in the viable cell count of air-blast dried yeast cells were used to assess the prolonged storability of starter product, depending on different concentrations of L-ascorbic acid or glutathione (1, 3, and 5 mM) as an additional protectant. These experiments examined validity after storage at 4 °C for two months (Fig. 2). The viable cell counts of *S. bacillaris* CD80, *M. caribbica* YP1, and *W. anomalus* CS7-16 decreased less than the control after the addition of antioxidants. Similarly, some *W. anomalus* SJ20 and *H. uvarum* SJ69 samples also exhibited lower reductions in viable cell counts than the control. However, *P. kluyveri* CD34 viability was not effected by antioxidant additives. *W. anomalus* SJ20 with 3 mM glutathione, *H. uvarum* SJ69 with 5 mM glutathione, *S. bacillaris* CD80 with 5 mM glutathione, *M.*

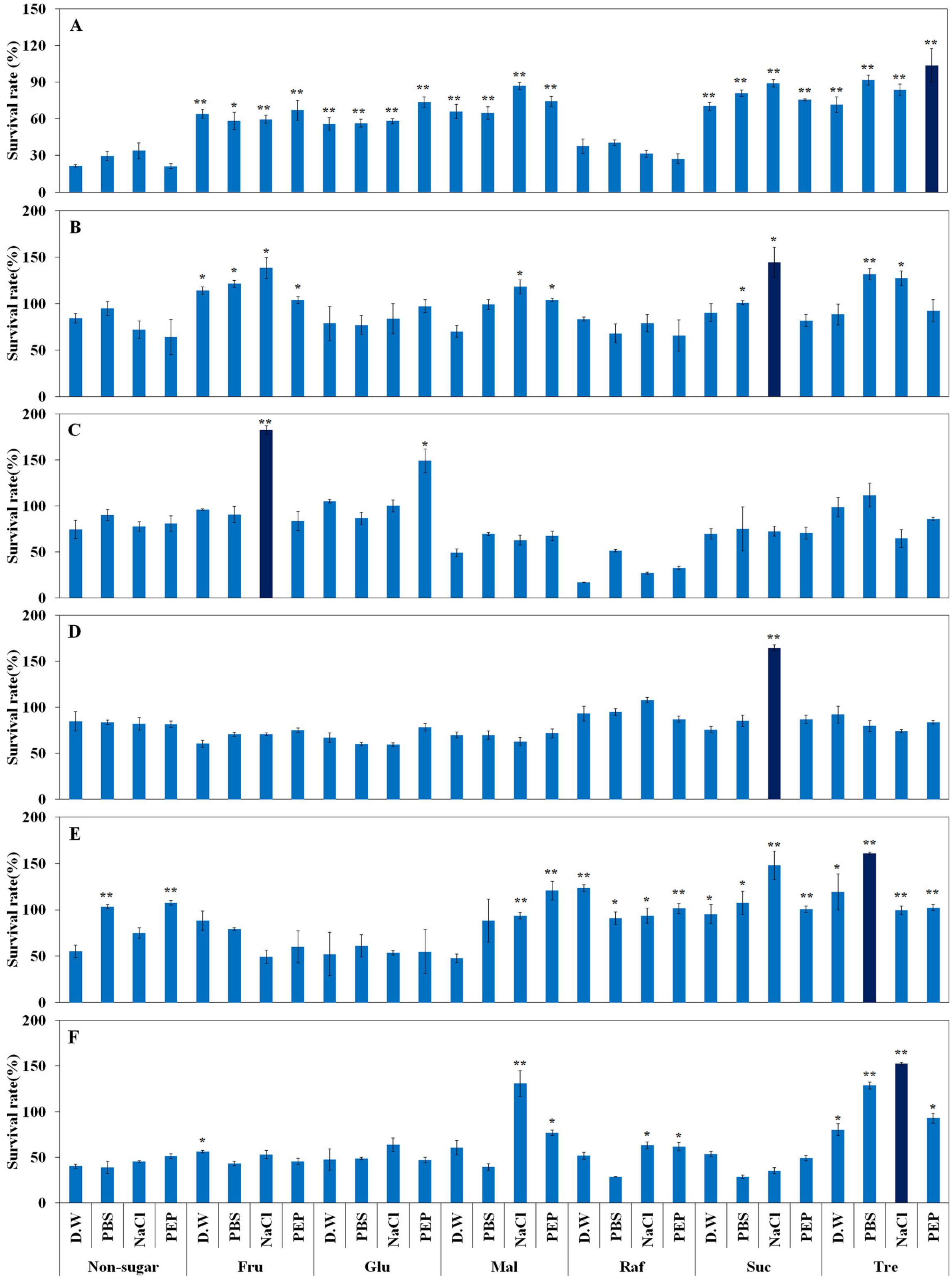

Fig. 1. Survival rates of air-blast dried *W. anomalus* SJ20 (A), *H. uvarum* SJ69 (B), *P. kluyveri* CD34 (C), *S. bacillaris* CD80 (D), *M. caribbica* YP1 (E), and *W. anomalus* CS7-16 (F), depending on sugar and rehydration solutions. Dark blue represents the best condition in each strain. All data are expressed as the means ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ are considered statistically significant using a Student's *t*-test. Fructose (Fru), glucose (Glu), maltose (Mal), raffinose (Raf), sucrose (Suc), and trehalose (Tre) were used as protectants and sterilized distilled water (D.W.), 1 × PBS buffer (PBS), 0.85% NaCl (NaCl), and 1% peptone water (PEP) were used as rehydration solutions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

caribbica YP1 with 5 mM L-ascorbic acid, and *W. anomalus* CS7-16 with 5 mM glutathione showed the highest preservative effects for viable cell counts among all antioxidant treatments, with a 0.29, 0.41, 0.90, 0.93, and 2.03 log reduction compared to control, respectively. In comparison, a study by Zhang et al. (2010) reported that glutathione showed a protective effect against cell membrane cryodamage by preventing the peroxidation of membrane fatty acids and protecting Na^{+}, K^{+}-ATPase when *Lactobacillus sanfranciscensis* DSM20451 cells were

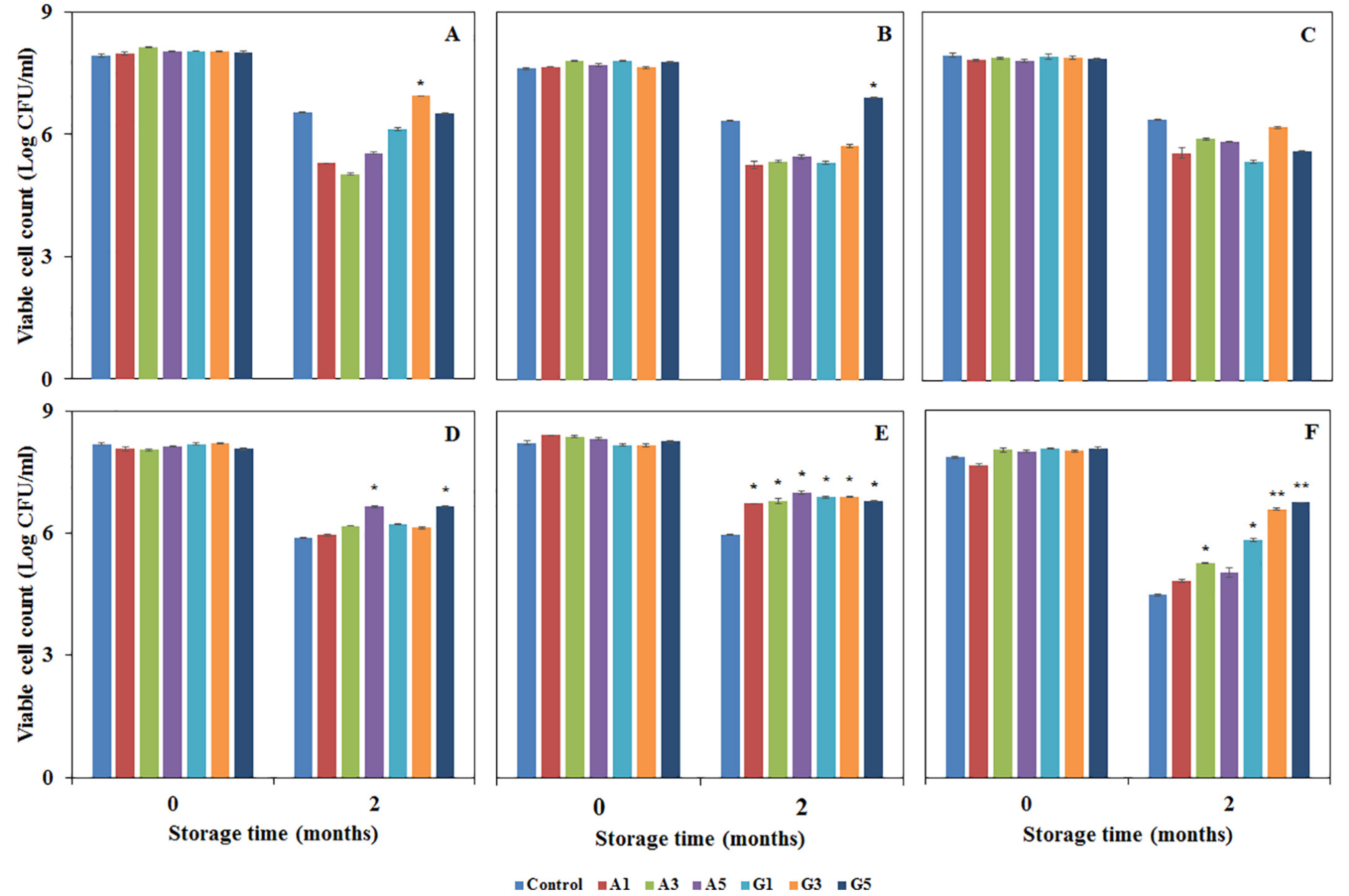

Fig. 2. Changes in the viable cell counts of air-blast dried *W. anomalus* SJ20 (A), *H. uvarum* SJ69 (B), *P. kluyveri* CD34 (C), *S. bacillaris* CD80 (D), *M. caribbica* YP1 (E), and *W. anomalus* CS7-16 (F), depending on different concentrations of Antioxidants. Legend A and G represent the different concentrations (1, 3, 5 mM) of L-Ascorbic acid and glutathione, respectively. Each sample was stored at 4 °C for two months before analysis. All data are expressed as the means ± SD (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$ are considered statistically significant using a Student's t-test and asterisks are indicated in case of only represented higher value compared to control.

subjected to freeze-drying. Moreover, Dave and Shah (1997) and Teixeira et al. (1995b) demonstrated that the viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* increased in the presence of L-ascorbic acid when prepared by freeze-drying and spray-drying, respectively.

The morphology of air-blast dried non-*Saccharomyces* yeast cells was examined using scanning electron microscopy (SEM; Fig. 3). These SEM images showed that the air-blast dried yeast cells were surrounded by various protectants and were compactly accumulated. In some images, bud scars were also observed using SEM (Fig. 3), which means air-blast drying stopped the proliferation of the yeast cells. Considering the rehydration results and morphology of air-blast dried yeast cells, we assumed that the increased survival rate (> 100%) was as a result of the re-initiation of the proliferation of the air-blast dried yeast cells during the 1-h rehydration. A study by Alegría et al. (2004) reported for the first time an increase in cell growth rate and cell size of lactic acid bacteria after lyophilization. In that study, 48% of the strains among the 43 freeze-dried *Oenococcus oeni* showed an average maximal population of 124.2 ± 57% and an average growth rate of 107.3 ± 43% compared to those of *O. oeni* before lyophilization. The authors suggested that those strains survived successfully and showed high growth rates by enlarging their morphology to overcome the cell death due to low temperatures and high vacuum.

3.6. Changes in the fermentation rate of air-blast dried non-Saccharomyces yeast cells

Fermentation rates of air-blast dried yeast cells depending on the storage period were examined to confirm the metabolic activities of the starter products (Fig. 4). Samples were prepared using the optimal manufacturing process determined for each yeast type in the study. Non-dried yeast cultures were used as controls to compare the fermentation rates of air-blast dried yeast cells between just after drying and following storage at 4 °C for two months (Lee et al., 2016a). As the survival rates of each of the air-blast dried cells after 1 h rehydration increased compared to the initial yeast cells prior to drying (Fig. 1), the fermentation rates of all air-blast dried yeast cells immediately following drying were determined to be slightly faster (or almost similar) than those of non-dried yeast cultures. In the case of air-blast dried yeast cells after two months of storage at 4 °C, the fermentation rates were slightly delayed compared to the controls because of a decrease in viable cell count during storage (Fig. 2). Although a slight delay in fermentation rate was found in the air-blast dried yeast cells stored at 4 °C for two months, it was not considered that it will substantially affect actual wine fermentation as this generally requires one to two weeks. Therefore, our results demonstrate the successful isolation of non-*Saccharomyces* yeast strains suitable for enhancing wine quality. We have also established an optimal 4 °C storage preservation condition, suggesting that the air-blast dried yeast cells developed in the study show strong potential as persimmon wine and apple cider starter products. These may contribute to improving wine quality in Korea and other countries, leading to further economic benefits.

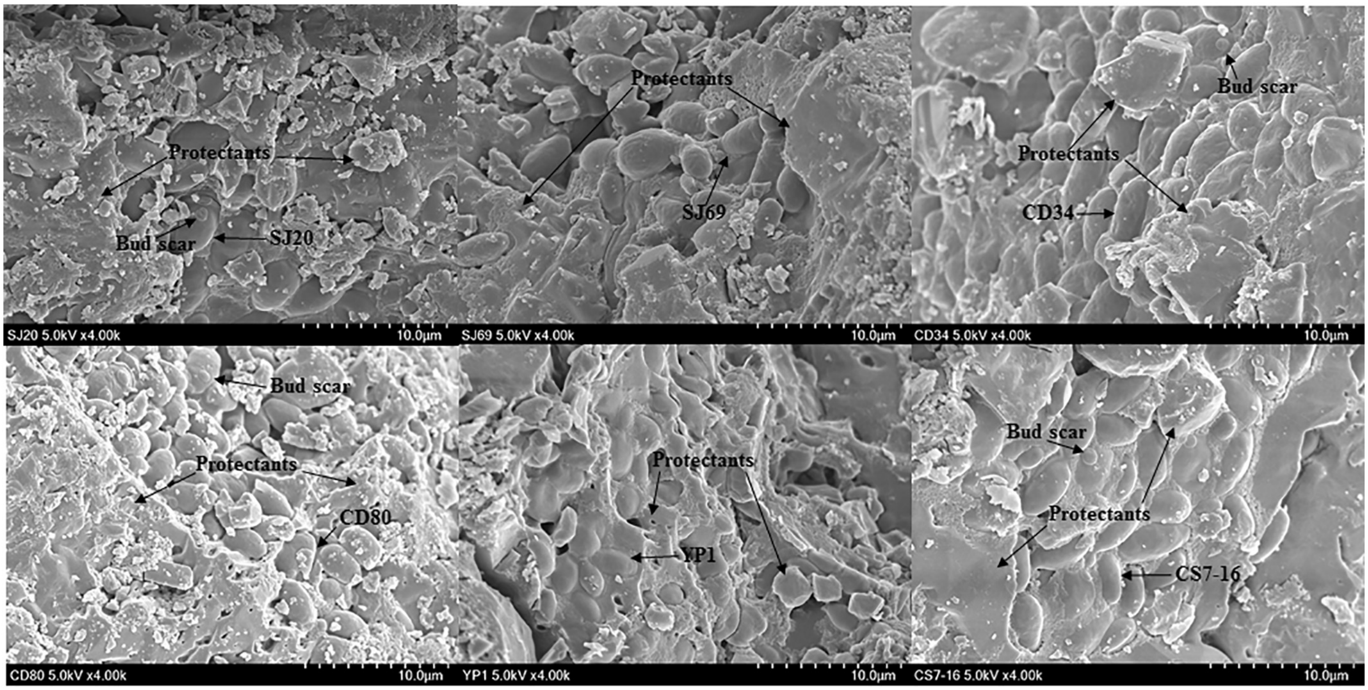

Fig. 3. Representative images of air-blast dried *W. anomalus* SJ20 (SJ20), *H. uvarum* SJ69 (SJ69), *P. kluyveri* CD34 (CD34), *S. bacillaris* CD80 (CD80), *M. caribbica* YP1 (YP1), and *W. anomalus* CS7-16 (CS7-16) collected using a scanning electron microscope (SEM) at 4000× magnification. All samples were air-blast dried with optimal sugar and antioxidant protectant concentrations obtained during the study; SJ20, trehalose and 3 mM glutathione; SJ69, sucrose and 5 mM glutathione; CD34, fructose and no antioxidant; CD80, sucrose and 5 mM glutathione; YP1, trehalose and 5 mM L-ascorbic acid; CS7–16, trehalose and 5 mM glutathione. Arrows represent protectants and each strain. Scale bars = 10 µm.

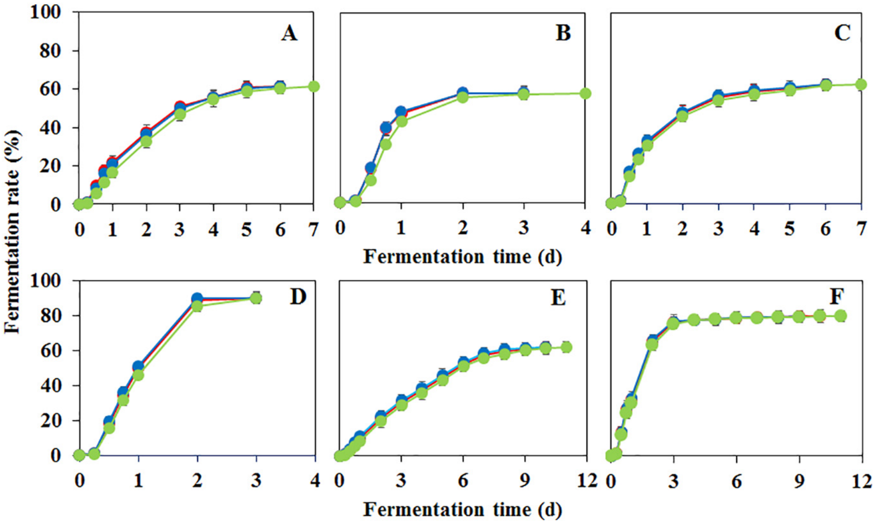

Fig. 4. The fermentation rates of air-blast dried *W. anomalus* SJ20 (circles), *H. uvarum* SJ69 (diamonds) (A), *P. kluyveri* CD34 (circles), *S. bacillaris* CD80 (diamonds) (B), *M. caribbica* YP1 (circles), and *W. anomalus* CS7-16 (diamonds) (C). Red figures represent non-dried yeast cells, blue figures represent air-blast dried yeast cells shortly after air-blast drying, and green figures represent air-blast dried yeast cells after two months of storage. All data are expressed as the means ± SD (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Declarations of conflicting interest

There are no conflicts of interest to declare.

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