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Fermentation Characteristics of Unripe *Citrus unshiu* Vinegar Production Using Acetic Acid Bacteria Isolated from Traditional Fermented Vinegars

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ABSTRACT: Here, we aimed to isolate an acetic acid bacterium that is suitable for the production of unripe *Citrus unshiu* vinegar from traditional fermented vinegars. We compared the halo sizes of isolates to select a strain with superior acetic acid production capabilities and selected *Komagataeibacter kakiaceti* P6 (P6) as the final strain. Using *Acetobacter pasteurianus* CY (CY) and *A. pasteurianus* KACC 17058 (KACC 17058) as controls, we analyzed the total phenolic compounds, total flavonoid content, antioxidant activities, and organic acids of the selected strain to verify its suitability for acetic acid fermentation. On the 30th day of the fermentation period, P6 showed a total acidity of 4.86%, which was higher than that of control groups (CY, 4.16%; KACC 17058, 4.01%). The total phenolic compounds, total flavonoid content, 1,1-diphenyl-2-picrylhydrazyl scavenging activity, and ferric ion reducing antioxidant power values significantly increased during fermentation with P6 compared with the initial *C. unshiu* wine, and no significant differences were observed from the vinegars produced by CY and KACC 17058. Moreover, organic acid analysis revealed that the unripe *C. unshiu* vinegar produced with P6 had an acetic acid content of 26.15 mg/mL, which was significantly higher than those produced with CY and KACC 17058, indicating that the P6 strain effectively produces acetic acid without adversely affecting other quality aspects during fermentation. In conclusion, the novel P6 strain is expected to be used as a starter for fermenting unripe *C. unshiu* vinegar, and its excellent acetic acid production capabilities suggest potential applications for other vinegars.

Keywords: acetic acid bacteria, *Citrus unshiu*, fermentation, *Komagataeibacter kakiaceti*, vinegar

INTRODUCTION

Fermented vinegar has traditionally been used as a seasoning to enhance the taste of various foods (Lee et al., 2019; De Leonardis et al., 2022). It is primarily produced using fruits and vegetables rich in nutritional components (e.g., amino acids, organic acids, phenols, vitamins, and minerals), and numerous studies have reported that these components can help with digestion, fatigue recovery, and diabetes and possess antiobesity and anticancer properties (Ousaaid et al., 2020; Özdemir et al., 2022). Recently, the incidence of various diseases has been continuously increasing with the increase in the elderly population; consequently, foods for wellbeing, including health functional foods and health protectants aimed at preventing chronic diseases such as cancer, diabetes, and cardiovascular diseases, are gaining increasing attention (Park et al., 2020).

In the Republic of Korea, the majority of *Citrus unshiu*, commonly known as mandarins, are produced on Jeju Island, with an annual production of about 560,000 Mg (Jang et al., 2004). *C. unshiu* contains about 60 types of bioactive compounds, including flavonoids, carotenoids, phenylpropanoids, and limonoids (Lee et al., 2022b), along with various nutrients (e.g., free sugars, organic acids, dietary fibers, vitamins, and minerals) (Ahn et al., 2007). Consequently, numerous preclinical and clinical trials have reported on the various physiological activities induced by antioxidants in *C. unshiu* peels and pulp (Ahn et al., 2007; Park et al., 2020; Lee et al., 2022a). In the past, unripe *C. unshiu* fruits, known as green tangerines, were largely considered to have no industrial value and were often discarded in large quantities (Yi et al., 2022). However, their nutritional value has been recognized recently, leading to increased sales through the internet and direct transactions, and their distribution is now allowed in Jeju Special Self-Governing Province, Republic of Korea (Lee and Joo, 2021).

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Unripe *C. unshiu* is rich in functional substances, including flavonoids, polyphenols, limonoids, and vitamin C (Shin and Lee, 2021), and various products (e.g., green tangerine marmalade and vinegar) that contain these substances have been marketed. Nevertheless, because of the antimicrobial activities of essential oils (e.g., limonene) and flavonoids (e.g., hesperidin and naringin), acetic acid fermentation using unripe *C. unshiu* is challenging compared with other fruits (Yi et al., 2014). The total acidity, which is a quality indicator of fermented vinegar, depends on the type of acetic acid bacteria used during the manufacturing process of vinegar; thus, the screening and utilization of excellent acetic acid bacteria are important (Park et al., 2005; Şengün et al., 2022).

This study aims to isolate and select an excellent acetic acid bacterial strain that is suitable for unripe *C. unshiu* vinegar production, analyze the fermentation characteristics during the fermentation process of vinegar, and investigate the strain's potential for developing high-quality unripe *C. unshiu* vinegar by analyzing the bioactive substance content, antioxidant activity, and organic acid composition of its vinegar.

MATERIALS AND METHODS

Strains and materials

Unripe *C. unshiu* was purchased in July 2020 from Ommapum Agricultural Co., Ltd., which is located in Jeju Special Self-Governing Province, and used to produce vinegar. The pulp and peel of unripe *C. unshiu* were separated by hand, and the juice obtained from squeezing was used for the experiments. Four types of commercially available traditional fermented vinegars were purchased from Goljaknara Research Institute Agricultural Co., Ltd.; Yeongdong Dried Persimmon Farming Association Co.; Yeongyang Green Food Co., Ltd.; and Hanhongsoon Agricultural and Fisheries and used as sources for isolation to select an appropriate acetic acid bacterium for unripe *C. unshiu* vinegar. The commercial *Saccharomyces cerevisiae* Fermivin (DMS Food Specialties) was used as yeast in the production of unripe *C. unshiu* wine. *Acetobacter pasteurianus* CY (KACC 92333p), which was previously isolated and preserved in the Food Microbiology and Biotechnology Lab at Kyungpook National University, and *A. pasteurianus* KACC 17058, which was obtained from the Rural Development Administration, were used as controls for the selected isolated strains.

Vinegar production

Fifteen kilograms of unripe *C. unshiu* was washed with baking soda (100% sodium bicarbonate, Church & Dwight Co., Inc.), peeled, and extracted to obtain the juice. Then, 10 L of unripe *C. unshiu* juice was placed in a 20-L sterilized fermentation container made of PET (height, 45 cm; diameter, 28 cm), equipped with an airlock, adjusted to 17.5°Brix sugar content, treated with 100 mg/L of $K_2S_2O_5$ (potassium metabisulfite), and left to stabilize for 2 h. Afterward, the prepared juice was inoculated with 0.02% (w/v) *S. cerevisiae* Fermivin. Subsequently, the mixture was fermented at 20°C for 5 days to produce unripe *C. unshiu* wine. To prepare the starter cultures for acetic acid fermentation, each control and isolated acetic acid bacterial strain were inoculated into 5% (v/v) YPM liquid medium (0.5% yeast extract, 0.3% peptone, and 2.5% D-mannitol) and cultured at 30°C for 48 h at 150 rpm. Then, they were added to sterilized unripe *C. unshiu* wine and incubated at 30°C for 10 days at 150 rpm. The starter culture and unripe *C. unshiu* wine were mixed at a 2:8 (v/v) ratio and subjected to acetic acid fermentation in a 5-L fermentation container at 30°C for 30 days to produce unripe *C. unshiu* vinegar.

Screening for acetic acid-producing bacteria

A 5% (v/v) inoculation was conducted in YPM liquid medium followed by cultivation at 30°C for 48 h at 150 rpm to isolate strains with excellent acetic acid production capabilities from four commercially available traditional fermented vinegars. Subsequently, streak plating was performed using a platinum loop, and 64 single colonies showing the characteristics of acetic acid bacteria were primarily screened. These colonies were then inoculated on GYC solid medium [3% glucose, 0.5% yeast extract, 1.0% $CaCO_3$, 3% (v/v) ethanol, and 1.5% agar] to observe the formation of clear zones. Based on the presence of clear zones, six colonies were selected for further analysis. To compare the acetic acid production capabilities of the six isolates, relative halo sizes were determined by comparing their clear zones and colony sizes on GYC solid medium (Guo et al., 2008), and the final acetic acid-producing strain was selected through comparison with control strains (*A. pasteurianus* CY and KACC 17058).

The 16S rRNA gene sequences of the final selected strain were analyzed and then compared with sequences recorded in the gene bank using the National Center for Biotechnology Information's Basic Local Alignment Search Tool. Multiple sequence alignment was performed using ClustalW in the BioEdit program (v7.2.5) (Thompson et al., 1997). Meanwhile, phylogenetic tree analysis was conducted using the neighbor-joining method in the MEGA (v6.06) program, and the reliability of branches within the molecular phylogeny was assessed using the bootstrap method with 1,000 replications (Felsenstein, 1981; Tamura et al., 2013).

Analysis of fermentation characteristics

The pH levels of fermentation cultures were measured using a pH meter (MP225K, Mettler-Toledo CH). The

supernatant of unripe *C. unshiu* vinegar was obtained by centrifugation at 4°C and 4,973 *g* for 15 min. To determine the total acidity, 10 mL of the supernatant was titrated with 0.1 N NaOH solution to pH 8.3 after adding 2~3 drops of 1% phenolphthalein, and the amount of 0.1 N NaOH consumed was converted into an organic acid coefficient equivalent to acetic acid (Sim et al., 2018). To measure the viable cell count, 1 mL of the vinegar sample was added with 9 mL of sterile distilled water. Next, the sample was diluted to the appropriate concentration using the serial dilution method and spread on YPM solid medium. Thereafter, it was incubated at 30°C for 48 h, and the growing colonies were counted as colony forming units (CFU/mL). The alcohol content was analyzed in accordance with the National Tax Service Liquor License Support Center (2017) regulations. After centrifugation, 100 mL of the supernatant was distilled to obtain 70 mL of distillate. Next, 30 mL of distilled water was added to this distillate, and after adjusting to 15°C, the alcohol content was measured using an alcohol meter and converted to alcohol by volume using the Gay-Lussac alcohol conversion table.

Analysis of the physicochemical properties of vinegar

The organic acid content was analyzed through high-performance liquid chromatography (Model Prominence, Shimadzu Co.) using a PL Hi-Plex H column (7.7×300 mm, Agilent Technologies) with a flow rate of 0.6 mL/min and temperature of 65°C. The mobile phase was 0.005 M sulfuric acid. The supernatant of unripe *C. unshiu* vinegar was filtered through a Sep-Pak C18 Plus Cartridge (Waters Co., Ltd.) and a 0.45-μm nylon syringe filter (SN02545, Lubitech Technologies Co., Ltd.) before analysis.

The total phenolic compound content was quantified colorimetrically in accordance with the Folin-Denis method (Amerine and Ough, 1980). One milliliter of 50% Folin-Ciocalteu phenol reagent was added to 1 mL of the supernatant of unripe *C. unshiu* vinegar, and the solution was left at room temperature for 3 min. Then, 1 mL of Na_2CO_3 solution was added, and the mixture was allowed to react in the dark at room temperature for 1 h before measuring the absorbance at 700 nm using a spectrophotometer. The total phenolic compound content was calculated using a standard curve prepared with gallic acid.

To determine the total flavonoid content, 430 μL of 50% ethanol and 50 μL of 5% sodium nitrite were added to 70 μL of the supernatant of unripe *C. unshiu* vinegar. The mixture was left at room temperature for 30 min. Then, 50 μL of 10% aluminum nitrate nonahydrate was added, and the reaction was allowed to proceed at room temperature for 6 min. After adding 500 μL of 1 N sodium hydroxide, the absorbance was measured at 510 nm using a spectrophotometer, and the total flavonoid content was calculated using a standard curve prepared with catechin (Zhishen et al., 1999).

Antioxidant activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of vinegar was measured in accordance with Blois' (1958) method. Fifty microliters of the supernatant of unripe *C. unshiu* vinegar was mixed with 150 μL of 0.1 mM DPPH solution in a 96-well plate and incubated in the dark at room temperature for 15 min. Distilled water and 100 μg/mL of ascorbic acid were used as negative and positive controls, respectively. The absorbance was measured spectrophotometrically at 517 nm (Benzie and Strain, 1996).

The ferric ion reducing antioxidant power (FRAP) assay was performed to assess the antioxidant capacity of vinegar. First, 25 μL of the supernatant of unripe *C. unshiu* vinegar was added to a 96-well plate. Then, a 10:1:1 (v/v/v) solution of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine, and 20 mM ferric chloride was prewarmed at 37°C for 15 min. Thereafter, 175 μL was added to each well of the plate. Then, the mixture was incubated in the dark at room temperature for 30 min, and the absorbance was measured at 590 nm. The results were converted to μg TE (Trolox equivalents)/g using a standard curve.

Statistical analysis

All experiments were conducted with at least three replicates, and the results are presented as mean±standard deviation. Analyses of variance and Duncan's multiple range tests were performed using SAS (version 9.4, SAS Institute Inc.) to determine the significance of the findings ($P<0.05$).

RESULTS AND DISCUSSION

Screening and identification of acetic acid-producing bacteria

Sixty-four potential isolates from four types of traditional fermented vinegar were primarily screened for the identification of excellent acetic acid-producing bacteria (data not shown). Utilizing the fact that acetic acid bacteria can dissolve $CaCO_3$ in GYC solid medium, forming a clear zone (Bang et al., 2022), six isolates forming clear zones were selected for secondary screening. *A. pasteurianus* CY and KACC 17058 were used as controls to compare the acetic acid production capability of these isolates, and the colony and clear zone sizes of eight strains, including the controls, were examined, comparing their acetic acid production ability through their relative halo sizes (Table 1). The relative halo sizes revealed that the P6 strain exhibited the highest relative halo size at 2.57, in-

Table 1. Colony size, clear zone, and relative halo size of each isolate

Strain	Colony size (cm)	Clear zone (cm)	Relative halo size[1)]
S1	0.41±0.10[b]	0.72±0.00[c]	1.75±0.07[c]
S2	0.42±0.10[b]	0.00±0.00[d]	0.00±0.00[e]
BR3	0.74±0.00[a]	1.41±0.10[b]	2.00±0.05[b]
P5	0.64±0.00[a]	1.31±0.10[b]	2.17±0.04[b]
P6	0.73±0.20[a]	1.83±0.00[a]	2.57±0.05[a]
R7	0.72±0.10[a]	1.44±0.00[b]	2.00±0.05[b]
Acetobacter pasteurianus KACC 17058	0.70±0.00[a]	1.31±0.10[b]	1.86±0.03[c]
A. pasteurianus CY	0.44±0.00[b]	0.64±0.10[c]	1.50±0.05[d]

Values are presented as mean±SD (n=3).
Different letters within the same column (a-e) indicate significantly different means (P<0.05).
[1)]Relative halo size = clear zone/colony size.

dicating that it had the highest acetic acid production capability. Ultimately, this strain was selected as the optimal fermentation strain for unripe *C. unshiu* vinegar. The analysis of the 16S rRNA gene sequence of the P6 strain showed 100% homology with the type strain *Komagataeibacter kakiaceti* JCM 25156 (Fig. 1).

Fermentation characteristics of unripe *C. unshiu* vinegar

The newly isolated *K. kakiaceti* P6 strain was inoculated into unripe *C. unshiu* wine with an initial alcohol concentration of 7.3% to verify its potential as a starter culture bacterium for vinegar production. For comparison, the control strains *A. pasteurianus* CY and KACC 17058, which were previously isolated from traditional fermented vinegar, were also inoculated into wine with the same initial alcohol concentrations. The fermentation characteristics of unripe *C. unshiu* vinegar during fermentation are shown in Fig. 2. As fermentation progressed, the pH generally decreased across all experimental groups, with the most significant reduction seen in P6 vinegar (Fig. 2A). This decrease in pH is attributed to acetic acid bacteria utilizing alcohol as an energy source and fermentation substrate to produce acetic acid (Bang et al., 2020). The total acidity serves as a vinegar quality indicator as acetic acid bacteria can break down alcohol to produce acetic acid and CO_2 (Kwon et al., 2014; Park et al., 2021). The total acidity contents of vinegars are shown in Fig. 2B. As the fermentation period increased, the total acidity increased in all experimental groups, with the highest value (4.86%) recorded on the 30th day of fermentation in the P6 group. According to Kim et al. (2001) who used kelp extract to produce vinegar, while pH decreases, the total acidity increases as ethanol, sugar, and other solution components are converted to acetic acid. Kim et al. (2020) reported that the total acidity increased to 7.3% when *A. pasteurianus* YJ17, which was isolated from brown rice, was used to ferment mulberry fruits. The total acidity of vinegar varies depending on the type of acetic acid bacteria. In the present study, the highest total acidity content was observed when the P6 strain was used, indicating that it is the most suitable strain for producing unripe *C. unshiu* vinegar.

The viable cell count results of unripe *C. unshiu* vinegars are shown in Fig. 2C. As fermentation progressed, the viable cell counts increased in all experimental groups, with the P6 group showing a higher rate of increase, surpassing the other strains by day 10 and reaching the highest viable cell count (7.18 log CFU/mL) at the end of fermentation.

The alcohol contents of unripe *C. unshiu* vinegars are shown in Fig. 2D. As the fermentation period increased, the alcohol content decreased in all experimental groups likely because of the conversion of alcohol to organic acids during the acetic acid fermentation process (Lee et

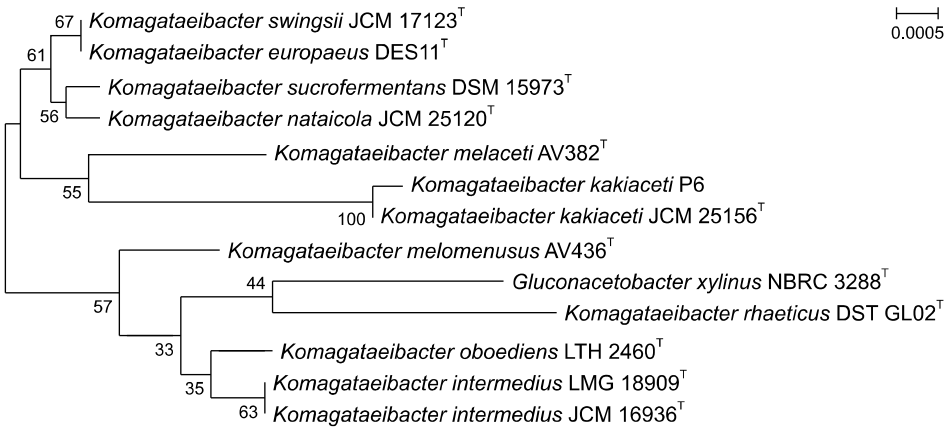

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of the P6 strain (the selected isolate) and related sequences. The related sequences were obtained using a Basic Local Alignment Search Tool search on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). All sequences were aligned using the Clustal X software. The tree was constructed using the neighbor-joining method and the Kimura two-parameter calculation model in MEGA 6.

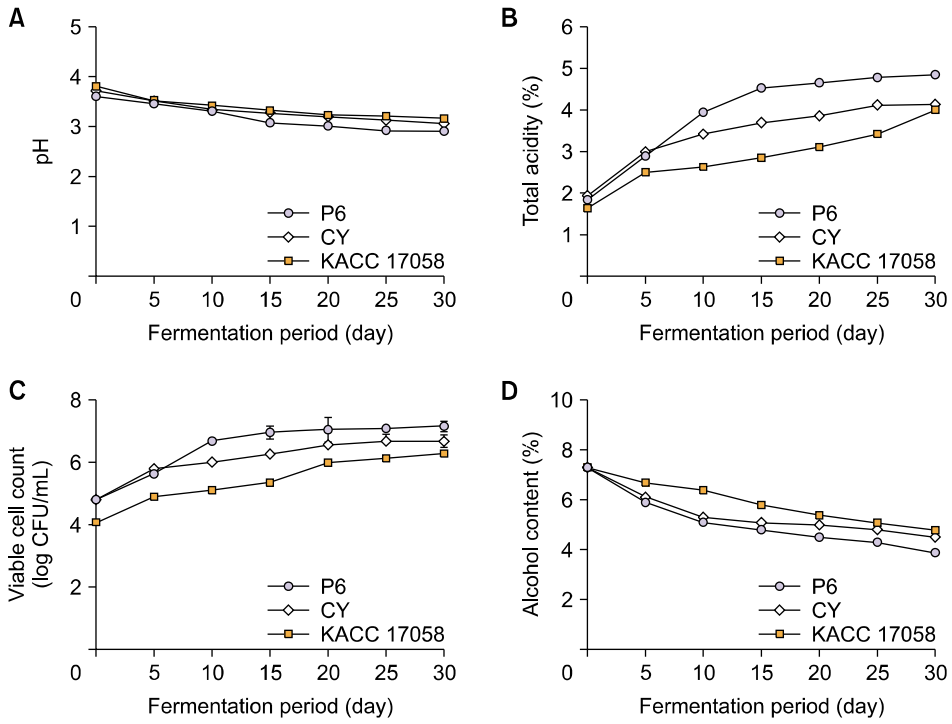

Fig. 2. Changes in pH (A), total acidity (B), viable cell count (C), and alcohol content (D) during the fermentation of unripe *Citrus unshiu* vinegar using three bacterial strains: *Komagataeibacter kakiaceti* P6 (P6), *Acetobacter pasteurianus* CY (CY), and *A. pasteurianus* KACC 17058 (KACC 17058).

al., 2012). Compared with other vinegars, the P6 vinegar exhibited the lowest alcohol content at the end of fermentation. As the P6 group showed the highest rate of reduction in alcohol and increase in total acidity during the acetic acid fermentation process, it was considered as the optimal fermentation strain for unripe *C. unshiu* vinegar.

Total polyphenol and flavonoid contents

Phenolic compounds, which are widely distributed in plants, are mostly secondary metabolites possessing various biological activities, including antioxidant, anticancer, and anti-inflammatory properties, because of their hydroxyl groups (Lee et al., 2005; Kim et al., 2009). Belonging to the polyphenol group, flavonoids are categorized into flavonols, flavanones, catechins, and isoflavones based on their chemical structure, which influences their biochemical activity (Kim et al., 2010). The unripe *C. unshiu* wine was analyzed as a control to observe the effect of each strain on the phenolic and flavonoid compounds during acetic acid fermentation. The total phenolic and flavonoid contents in unripe *C. unshiu* vinegar are shown in Fig. 3. Unripe *C. unshiu* wine contained the lowest total phenolic compound content (0.98 mg/mL), and all vinegar samples exhibited a significant increase in phenolic content compared with the control, showing approximate increases of 30%~40%. In addition, all vinegar samples contained higher total flavonoid content than wine, with the P6 group showing the highest value at 0.053 mg/mL, approximately 32.5% higher than that of the control. Similarly, Gao et al. (2022) observed changes in phenolic compounds and flavonoid contents during the acetic acid fermentation of black wolfberry. They found that the total phenolic compounds and flavonoid contents increased as fermentation progressed.

Several studies have examined bioactive compounds in vinegars created by fermentation using the same or similar fruits. In Yi et al.'s (2014) study, which analyzed the total phenolic and flavonoid contents in vinegar made from immature and ripe citrus fruits, immature citrus vinegar showed an approximately 6.7-fold increase in total phenolic compounds and a greater than 5.7-fold increase in flavonoid content compared with vinegar made from ripe fruits. According to Park et al. (2020), premature

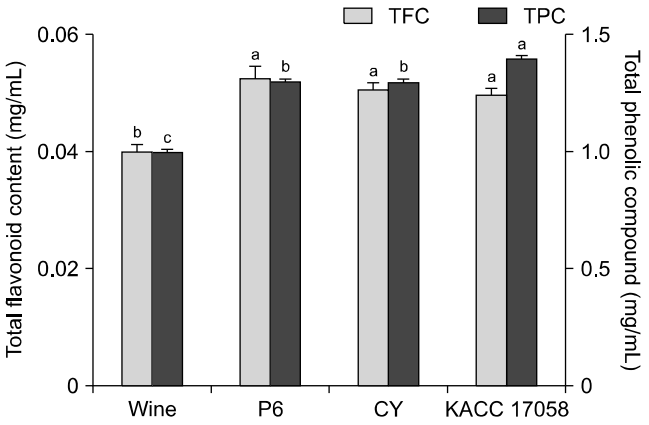

Fig. 3. Total phenolic compounds (TPC) and total flavonoid contents (TFC) of unripe *Citrus unshiu* vinegars and unripe *C. unshiu* wine that was used to make them. Three bacterial strains were used for fermentation: *Komagataeibacter kakiaceti* P6 (P6), *Acetobacter pasteurianus* CY (CY), and *A. pasteurianus* KACC 17058 (KACC 17058). Different letters above the bar (a-c) mean scores that are significantly different (P<0.05) by Duncan's multiple range test.

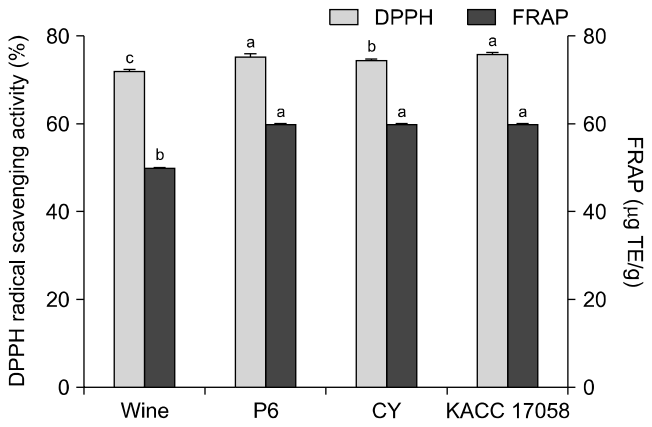

Fig. 4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and ferric ion reducing antioxidant power (FRAP) of unripe *Citrus unshiu* vinegars and unripe *C. unshiu* wine that was used to make them. Three bacterial strains were used for fermentation: *Komagataeibacter kakiaceti* P6 (P6), *Acetobacter pasteurianus* CY (CY), and *A. pasteurianus* KACC 17058 (KACC 17058). Different letters above the bar (a-c) mean scores that are significantly different (P<0.05) by Duncan's multiple range test.

mandarin vinegar had a higher flavonoid content (3 μg CE/mL) than vinegars made from premature mandarin mixed with 10% dried or roasted Citri Unshius Pericarpium Immaturus. Lee et al. (2014) studied the production of rice vinegar with the addition of *Akebia quinata* fruit during fermentation. They found that the total polyphenol and flavonoid contents in vinegar increased with increasing amount of *A. quinata* fruit and the progression of acetic acid fermentation. These changes in bioactive substances are because of the conversion of bound polyphenolic compounds in the fermentation substrate to free forms by microbe-derived acids, sugars, or lipolytic enzymes during the fermentation process (Cho et al., 2017).

Antioxidant activity

The antioxidant activities of unripe *C. unshiu* vinegars are shown in Fig. 4. With regard to DPPH radical scavenging activity, unripe *C. unshiu* wine showed an activity of 72.08%, and all vinegar samples exhibited an increase because of acetic acid fermentation, with the KACC 17058 group showing the highest activity (76.08%). The FRAP assay produced similar results, wherein all vinegar samples showed significant increases in antioxidant activity compared with the control (50 μg TE/g). Yi et al. (2014) analyzed the DPPH radical scavenging activity of vinegar made from immature and ripe *C. unshiu*. They reported that immature *C. unshiu* vinegar showed greater DPPH radical scavenging activity and higher total phenolic and flavonoid contents than ripe *C. unshiu* vinegar. Chen et al. (2017) analyzed the changes in DPPH radical scavenging activity in citrus vinegar, which underwent primary alcohol fermentation with *S. cerevisiae* (dry yeast) and *Lactobacillus plantarum* AS1.555 and secondary acetic acid fermentation with *A. pasteurianus* AS1.41. They found that, along with an increase in polyphenol content, the DPPH radical scavenging activity increased from 25.5% to 37.4% with the progression of acetic acid fermentation. In another study, the DPPH radical scavenging activity increased in kombuchas brewed with various fruit peels as fermentation progressed, which was attributed to the increase in polyphenol and flavonoid contents (Lee and Yi, 2023). In their study comparing the antioxidant activities of 10 commercially available fermented vinegar products in Korea, Pyo et al. (2021) found that citrus vinegar had high polyphenol and flavonoid contents and FRAP antioxidant activity. The high FRAP values were attributed to the high content of quercetin derivatives and polyphenols. In another study that analyzed the FRAP activity of commercially available fruit juices, Lee et al. (2008) reported high FRAP values in orange and grapefruit juices (117.62 and 115.72 μM TE, respectively). In addition, these juices contained significantly higher DPPH radical scavenging activity and FRAP antioxidant activity compared with others, noting that the two values were significantly correlated.

Organic acid contents

The organic acid contents of unripe *C. unshiu* vinegars are shown in Table 2. Five types of organic acids were detected, with acetic acid and citric acid having the highest concentrations. Citric acid, which is primarily found in citrus juices (Song et al., 1998), had high concentrations even after acetic acid fermentation. The acetic acid contents in unripe *C. unshiu* vinegars produced with *K. kakiaceti* P6 and *A. pasteurianus* CY were 26.15 and 20.52 mg/mL, respectively, representing the highest organic acid content in these vinegars. Similarly, Park et al. (2020)

Table 2. Organic acid contents of unripe *Citrus unshiu* vinegars produced by different bacterial strains

Strain	Organic acid (mg/mL)				
	Citric acid	Malic acid	Succinic acid	Lactic acid	Acetic acid
Komagataeibacter kakiaceti P6	12.00±0.02^{b}	2.72±0.02^{c}	1.55±0.03^{b}	0.11±0.02^{a}	26.15±0.02^{a}
Acetobacter pasteurianus KACC 17058	14.58±0.01^{a}	3.80±0.03^{a}	1.81±0.02^{a}	0.08±0.01^{b}	11.63±0.04^{c}
A. pasteurianus CY	11.84±0.03^{c}	3.41±0.03^{b}	1.52±0.00^{c}	0.11±0.01^{a}	20.52±0.02^{b}

Values are presented as mean±SD (n=3).
Different letters within the same column (a-c) indicate significantly different means (P<0.05).

found that when acetic acid fermentation was conducted using premature mandarins harvested in July, the organic acids in the resulting vinegar contained (in descending order) acetic (13,288 mg/L), citric, succinic, and tartaric acids. In their study comparing organic acids found in vinegars produced with 30%, 35%, and 40% immature *C. unshiu* juice in 5% fermented alcohol, 5% seed vinegar, and distilled water, Yi et al. (2014) found that lactic acid and acetic acid were the most abundant organic acids, with the latter having the highest concentration (3,990 mg%) in the group with 40% immature *C. unshiu* juice. Additionally, Yi et al. (2017) reported that six types of organic acids were detected in lemongrass vinegar, with acetic acid being the predominant organic acid (3,658.58 mg%). The present study found that unripe *C. unshiu* vinegars fermented with *K. kakiaceti* P6 and *A. pasteurianus* CY also showed significant amounts of acetic acid, indicating that normal acetic acid fermentation occurred.

In this study, while all unripe *C. unshiu* vinegars exhibited similar increases in antioxidant activity, different organic acid contents, total acid production, and polyphenol contents were observed when using three types of acetic acid bacteria, including a novel isolate (*K. kakiaceti* P6). This variation can be attributed to differences in metabolic and enzyme activities, leading to different substrate degradation characteristics when different acetic acid bacteria were used (Chen et al., 2022). In conclusion, the novel strain *K. kakiaceti* P6 demonstrated higher acid production and physiological activity characteristics during vinegar fermentation than either *A. pasteurianus* strain, indicating its high potential as a starter culture bacterium for unripe *C. unshiu* vinegar.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: SHW, HDP, SBL. Analysis and interpretation: SHW, YJK, KTC, JSC, SBL. Data collection: SHW, YJK, SBL. Writing the article: SHW, YJK, SBL. Critical revision of the article: HDP, SBL. Final approval of the article: all authors. Statistical analysis: KTC, JSC, SBL. Obtained funding: SBL. Overall responsibility: SHW, HDP, SBL.

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