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Screening of Acetic Acid Bacteria Isolated from Various Sources for Use in Kombucha Production

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Abstract: The objective of this study was to isolate and identify strains of *Acetobacter* suitable for use in the development of a complex microbial culture for producing Kombucha and to examine the fermentation characteristics for selection of suitable strains. A medium supplemented with calcium carbonate was used for isolation of acetic acid bacteria from 22 various sources. Colonies observed in the clear zone resulting from decomposition of calcium carbonate by acid produced by microorganisms were collected. Identification of the collected strains was based on biological and morphological characteristics, and the results of base sequence analysis. A total of 37 strains were identified, including six species in the *Acetobacter* genus: *Acetobacter pasteurianus*, *Acetobacter orientalis*, *Acetobacter cibinongensis*, *Acetobacter pomorum*, *Acetobacter ascendens*, and *Acetobacter malorum*, as well as one species in the *Gluconobacter* genus, *Gluconobacter oxydans*. Among thirty-seven strains, seven strains of acetic acid bacteria with exceptional acid and alcohol tolerance were selected, and an evaluation of their fermentation characteristics according to fermentation temperature and period was performed. The results showed a titratable acidity of 1.68% for the *Acetobacter pasteurianus* SFT-18 strain, and an acetic acid bacteria count of 9.52 log CFU/mL at a fermentation temperature of 35 °C. The glucuronic acid and gluconate contents for the *Gluconobacter oxydans* SFT-27 strain were 10.32 mg/mL and 25.49 mg/mL, respectively.

Keywords: Kombucha; *Acetobacter*; *Gluconobacter*; glucuronic acid; fermentation characteristics

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1. Introduction

The oxidative fermentation capacity of acetic acid bacteria (AAB) is known to involve an incomplete oxidation process where the substrate is oxidized by dehydrogenase, leading to release of the resulting oxidized product [1]. Nineteen genera of AAB, including *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and others, have been recognized based on the results of genetic analysis and their respective characteristics [2]. The presence of AAB has been detected in a variety of foods; *Acetobacter aceti* (*A. aceti*), *Acetobacter pasteurianus* (*A. pasteurianus*), *Acetobacter malorum* (*A. malorum*), and *Acetobacter pomorum* (*A. pomorum*) are the most frequently isolated species in the process of vinegar fermentation [3,4].

Growth of *Gluconobacter* (*Glu.*), a Gram-negative, rod-shaped acetic acid bacterium, can cause incomplete oxidization of a wide range of carbohydrates and alcohols, which can occur in highly concentrated sugar solutions and at low pH. *Gluconobacter* is used extensively in industrial processes in the production of gluconic acid from glucose and sorbose from L-sorbitol [5]. *A. aceti*, *A. pasteurianus*, *Glu. europaeus*, *Glu. hanseni*, and *Glu. oxydans* species have received approval from the Korean Ministry of Food and Drug

Safety as generally recognized as safe (GRAS) food materials for use in the production of vinegar [6,7].

Acetic acid bacteria and gluconic acid-producing bacteria, mainly *Komagataeibacter xylinus* (*K. xylinus*), *Bacterium. gluconicum*, *A. aceti*, *A. pasteurianus*, *A. musti*, *Glucobacter oxygendans* (*G. oxygendans*), and *Glu. potus*, are the dominant prokaryotes found in Kombucha cultures [8]. Among them, an association of *K. xylinus* with the production of cellulose biofilms floating on the surface of tea broth in Kombucha has been reported [9].

Kombucha, a fermented beverage, is produced by introducing a symbiotic culture of bacteria and yeast (SCOBY) into a mixture created by combining sugar with water brewed from green or black tea. This beverage was reportedly administered in Ancient China as a remedy for various infirmities during the period of Emperor Qin Shi Huangdi, and it is believed that it was first distributed from Russia to Eastern Europe, traveling by trade routes, and gained popularity in Germany during the 19th century and then expanded to European countries [10,11].

Metabolism of microorganisms by a SCOBY, a cellulose biofilm formed during fermentation of Kombucha, occurs in the production of a variety of functional substances during the process of Kombucha fermentation. In addition, use of SCOBY has been attempted in various fields of active research, not only for medical applications that better support high-water holding capacity and strength compared to the properties of plant cellulose, but also in a range of commercial applications through the synthesis of bioactive compounds containing bacterial cellulose with fine structures [12]. A SCOBY, composed of a mixture of bacteria and yeast used in the preparation of foods and beverages, contains particular genera of bacteria and yeasts, including *Gluconobacter*, *Acetobacter*, *Zygosaccharomyces*, *Saccharomyces* sp., and *Schizosaccharomyces* [13]. In the process of symbiotic fermentation, yeast is responsible for converting sugar into alcohol, while acetic acid bacteria utilize alcohol and sugar to produce acetic acid and gluconic acid [14,15].

The production of Kombucha involves fermentation through cooperation of specific bacteria and yeast, using a SCOBY composed of various species of bacteria and yeast [16]. The flavor profile of Kombucha is significantly influenced by the resulting microbial compositions and fermentation conditions. In addition, this process of fermentation can yield substances that include polyphenols, amino acids, organic acids (including acetic acid, gluconic acid, and glucuronic acid), minerals, vitamins, and D-saccharic acid 1,4-lactone (DSL), which contribute to its proven health benefits, including antioxidant effects, promotion of digestion, skin health, antimicrobial properties, and others [17,18].

Despite extensive research on the efficacy and marketability of Kombucha, focus on the development of standardized manufacturing methods has been limited. This includes use of fermentation techniques that can ensure consistent culture time, temperature, substrate, and additive parameters. In particular, the preparation of Kombucha is currently reliant on the use of imported Kombucha powder and SCOBY starter.

These are important considerations because various variables are dependent on microbial composition. Particularly during fermentation, the challenge of producing exceptional fermented products with consistent functionalities (including gluconic acid, glucuronic acid content, and antioxidant activity, etc.) is more complex. Therefore, the objective of this study was to identify isolated strains suitable for the composition of acetic acid bacteria among complex microbial cultures, which are major bacterial components.

2. Materials and Methods

2.1. Materials

Vinegar starter, plum extract, and wine (Suncheon, Republic of Korea) were supplied by the Food Fermentation Engineering Laboratory, Department of Food Engineering, Sunchon National University. Nine types of fruits (Suncheon, Republic of Korea) were obtained from Suncheon Agricultural Products Wholesale Market, and the collection of bacteria from the surface of fruit was performed using a 3M Pipette Swab Plus^{+} (3M Korea Ltd., Seoul, Republic of Korea). Eight types of commercial fruit vinegars (Jangseong and

Namwon, Republic of Korea) and commercially available Kombucha (Masontops, North York, ON, Canada) were purchased for use as samples in the isolation of acetic acid bacteria.

2.2. Reagents

Yeast extract (Life Technologies Co., Miami, FL, USA), D-(+)-Glucose (Sigma-Aldrich Co., Louis, MO, USA), $CaCO_3$ (Taekyung Bk Co., Seoul, Republic of Korea), mannitol (Junsei Chemical Co., Chuo-ku, Tokyo), peptone (Duksan Pure Chemical Co., Ansan-si, Republic of Korea), and ethyl alcohol anhydrous (Daejung, Siheung-si, Republic of Korea) were purchased for use in preparation of medium. The medium used for isolation and selection of acetic acid bacteria contained YGCE agar (1.0% Yeast extract, 5.0% Glucose, 2.5% $CaCO_3$, 4.0% Ethanol, 2.0% Agar,) and MA agar (0.5% Yeast extract, 2.5% Mannitol, 0.3% Peptone, 1.0% $CaCO_3$, 1.5% Agar). The medium used for screening the most suitable strains according to fermentation characteristics contained YGE broth (1% Yeast extract, 5% Glucose, 3% Ethanol).

2.3. Isolation, Screening, and Identification of the Most Suitable Strains of Acetic Acid Bacteria

2.3.1. Isolation of Acetic Acid Bacteria

The 22 collected samples diluted with 0.85% NaCl were spread on isolation plate medium (YGCE agar and MA agar), 200 µL each, followed by incubation at 30 °C for three days. Isolation of pure bacterial strains from subcultures was repeated three times. This process was based on formation of clear zones around the colony, which could be easily observed by the naked eye [19]. The isolated strains were transplanted onto slant agar medium (1.0% Yeast extract, 5.0% Glucose, 4.0% Ethanol, 2.0% Agar) and used in experiments for selection of the most suitable strain.

2.3.2. Screening and Identification of Acetic ACID Bacteria

Screening of acetic acid bacteria was based on morphological and biological characteristics. Gram staining and simple staining were performed for microscopic examination to determine the morphology [20]. For biological evaluation, one drop of $FeCl_3$ solution was added to 1 mL of strain culture solution for testing of gluconic acid based on change of color from yellow to dark brown (Figure 1). For the catalase test, bacterial isolates were obtained from the surface of a sterilized glass slide using a loop according to the Reiner [21] method, followed by addition of one drop of 3% hydrogen peroxide for detection of bubbles (O_2 + water = bubbles).

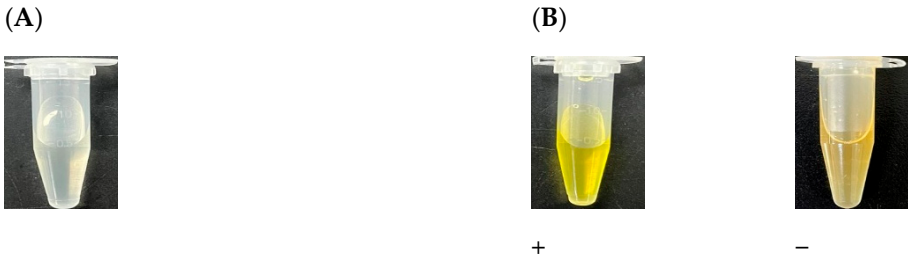

Figure 1. Changes in gluconic acid test of Gluconobacter strains. (**A**) Before reaction. (**B**) After reaction (+, positive; −, negative).

Colony PCR was performed according to the method reported by Wan et al. [22] using 785F (5′-GGA TTA GAT ACC CTG GTA-3′) and 907R (5′-CCG TCA ATT CMT TTR AGT TT-3′) primers, and sequencing of 16S ribosomal RNA in the screened bacterial colony was requested from Macrogen Inc. (Seoul, Republic of Korea) for confirmation of both forward (5′) and reverse (3′) directions. The analyzed DNA sequences were inserted into the BLAST (Basic Local Alignment Search Tool) program provided by the NCBI (National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)(accessed on 17 January 2023) for comparison with a search of the sequence database for identification of homologous sequences and to determine the systematical genetic relationship [23].

2.4. Selection of the Most Suitable Strains According to Fermentation Characteristics

2.4.1. Measurement of pH and Titratable Acidity (TTA)

The pH values for each strain were measured in 10 mL of culture media using a pH meter (HM-40X, Dkk-toa Co., Shinjuku-ku, Tokyo, Japan). The total amount of acid was shown as the percentage of acetic acid (%) after calculating the amount of solution used in neutralizing 2 mL of supernatant obtained from the centrifuged sample using 0.1 N NaOH until reaching a pH level of 8.3 after addition of 2–3 drops of 1% phenolphthalein [24].

2.4.2. Acid Resistance

YGE broth was used as the medium for determining the resistance level of the strains at various concentrations of acid. 1 N HCl and 1 N NaOH was added to the YGE broth for adjustment of the pH range to 4.0–8.0. pH-adjusted YGE broth was inoculated with 1% of each target strain, followed by culture at 30 °C. Absorbance was measured at 660 nm using a microplate reader (SPECTROstarNano, BMG Labtech, Ortenberg, Germany) for determination of growth rates according to incubation periods, which were presented as a percentage (%) compared to the controls.

2.4.3. Alcohol Tolerance

YG broth, consisting of 1% yeast extract and 5% glucose, was used as the medium for evaluating the tolerance level of the strains at various concentrations of alcohol. Ethyl alcohol anhydrous (Daejung, Siheung-si, Republic of Korea) was added for adjustment of the alcohol concentration of the YG broth to 2.0–10%. YG broth with the adjusted concentration of ethanol was inoculated with 1% of each target strain, followed by culture at 30 °C. Absorbance was measured at 660 nm using a microplate reader (SPECTROstarNano, BMG Labtech, Ortenberg, Germany) for determination of growth rates according to incubation periods, which were presented as a percentage (%) compared to the controls.

2.5. Viable Cell Count of Acetic Acid Bacteria

A standard plate count (SPC) was used for counting the number of viable cells in acetic acid bacteria according to fermentation temperature and period. Dilution of each sample with sterile diluent (0.85% NaCl) was performed in a step-by-step manner using the decimal dilution method, followed by plating of 1 mL of each diluted sample on YGE agar medium and incubation at 30 °C for three days. The average number of colonies was determined from the results of three independent experiments for calculation of the colony count, which was expressed as log CFU (colony forming units)/mL [25,26].

2.6. Content of Gluconate and Glucuronic Acid

Measurement of the gluconate and glucuronic acid content was performed using a modified version of the method reported by Ansari et al. [27]. The culture solution was centrifuged at 1000 rpm for 3 min (HA-1000-3, Hanil Science Industrial Co., Ltd., Incheon, Republic of Korea), followed by filtering of the supernatant through a 0.45 μm membrane filter (PVDF 25 mm, Chromdisc, Daegu, Republic of Korea), and analysis was then performed using HPLC (Waters 1525 and 717, Waters Co., Milford, MA, USA). A Supelcogel c-610h column (30 cm × 7.8 mm, Supelco, Bellefonte, PA, USA) was used with an oven temperature of 30 °C. The mobile phase was composed of 0.1% phosphoric acid with a flow rate of 0.5 mL/min. UV detection was measured at 210 nm using a Waters 996 detector (Waters Co., Milford, MA, USA). Sodium gluconate and D-glucuronic acid (Sigma-Aldrich Co., Louis, MO, USA) were used as standard reference materials (SRMs) and the content was presented using the external standard method.

2.7. Statistical Analysis

For statistical analyses, the experiments were repeated three times or more and analysis of the data was performed using IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY,

USA). Calculations of Mean ± SD and testing for significant difference of mean values were performed using Duncan's multiple range test ($p < 0.05$).

3. Results and Discussion

3.1. Isolation, Screening, and Identification of the Most Suitable Strains for Production of Acetic Acid and Gluconic Acid Bacteria for Kombucha Fermentation

3.1.1. Isolation and Selection of Acetic Acid Bacteria

For isolation of acetic acid bacteria, the 22 collected samples were spread on YGCE and MA agar media and 42 pure strains were isolated according to the size of clear zones formed around the colony (Figure 2). The morphological and biological characteristics of the isolated pure strains are shown in Table 1. Morphologically, most of the isolated strains were Gram-negative bacilli. FPA-4, FPP-1, FPP-3, FPP-4, and FPS-3 were identified as Gram-positive streptococci. The color of colonies was brown in most strains, while a white color was observed in colonies produced by FPA-4, FPP-1, FPP-3, FPP-4, and FPS-3. Regarding biological characteristics, the negative decomposition ability of mannitol was observed in FPP-1, FPP-3, FPP-4, and FPS-3 and a positive result was obtained from the remaining strains. A negative result was obtained for biofilm formation and the catalase-test in FPA-4, FPP-1, FPP-3, FPP-4, and FPS-3, and a positive result was obtained from the remaining strains. Strains FPA-4, FPP-1, FPP-3, FPP-4, and FPS-3 were identified as lactic acid bacteria based on the white colony color and negative results on the Gram-positive and catalase test [28]. The remaining strains exhibited characteristics identical to those of *Acetobacter* sp. (Figure 3) including Gram-negative, bacillus, obligate aerobe, and biofilm formation [29].

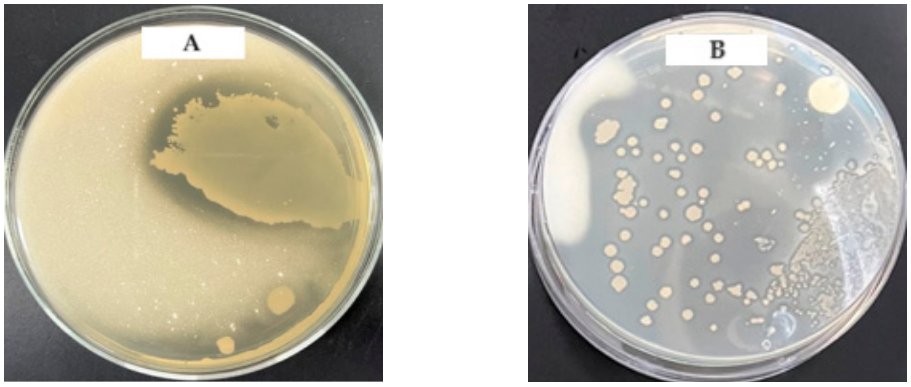

Figure 2. Isolated strains formed clear zones around the colony. (**A**) Clear zone of SMC-4 strain. (**B**) Clear zone of VVJ-2 strain.

The production of 5-keto- and 2-ketogluconic acids by strains of *Gluconobacter* is known to occur by partial oxidation of the carbon source (D-glucose) and alcohol. A dark yellow color was observed for gluconic acid, with Fe^{3+} oxidation-reduction in iron (II) ions of iron (III) chloride by the hydroxy group [30,31]. The result of the gluconic acid test was positive only for VVJ-1 and VVJ-2, which were identified as *Glucobobacter* sp. based on positive results on the catalase-test and the presence/absence of biofilm formation [32,33]. Following isolation and selection of acetic acid bacteria, 37 out of 42 species were confirmed as strains of acetic acid bacteria and sequencing of their 16S rDNA gene was performed.

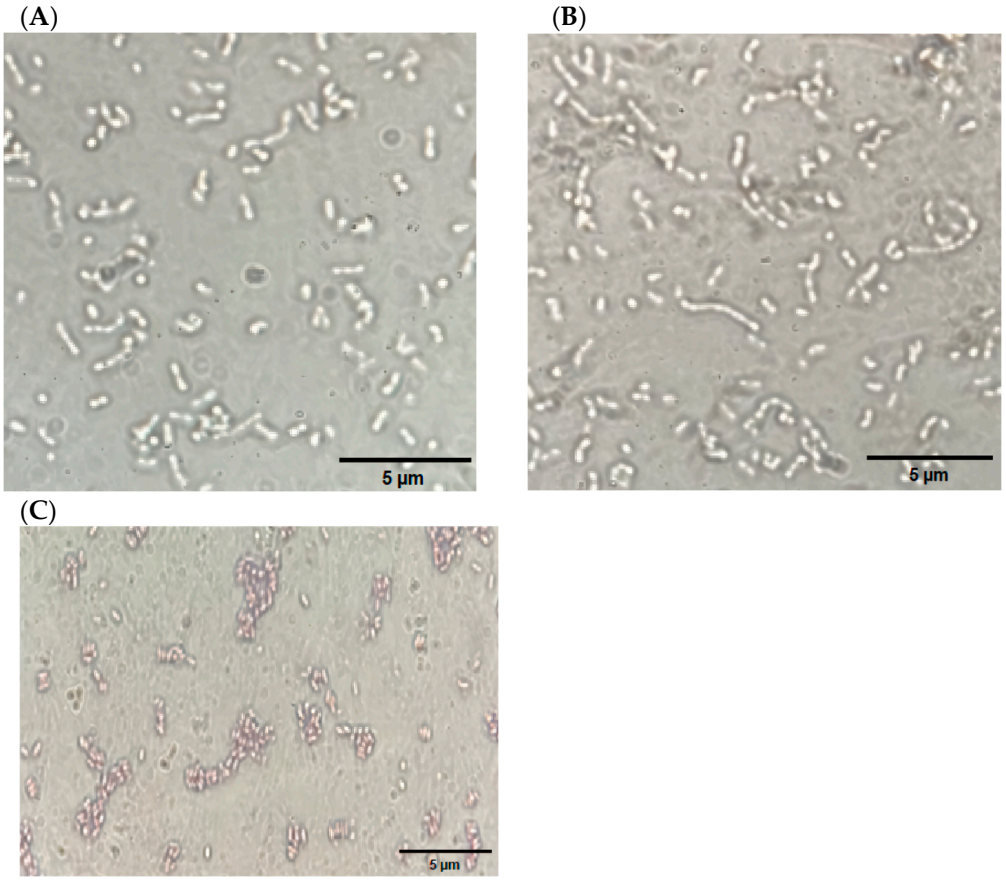

Figure 3. Cell characteristics of strains isolated from domestic fermented foods and produce. (**A**) Microscopic examination of SMC-4 strain, (**B**) microscopic examination of VVJ-2 strain, (**C**) Gram-negative reaction result of acetic acid bacteria.

Table 1. Morphological, biological, and fermentation characteristics of 42 strains isolated from collected samples.

Isolate	Morphological		Biological			D-Mannitol Assimilation
	Colony Morphology	Gram Staining	Gluconic Acid Test	Biofilm Formation	Catalase Test	
SVC-04	rod-shaped, light brown	−	−	+	+	+
SVC-12	rod-shaped, light brown	−	−	+	+	+
SVC-14	rod-shaped, light brown	−	−	+	+	+
SVC-22	rod-shaped, light brown	−	−	+	+	+
SVC-38	rod-shaped, light brown	−	−	+	+	+
SVC-49	rod-shaped, light brown	−	−	+	+	+
SVC-410	rod-shaped, light brown	−	−	+	+	+
SVC-54	rod-shaped, light brown	−	−	+	+	+
SMC-1	rod-shaped, light brown	−	−	+	+	+
SMC-2	rod-shaped, light brown	−	−	+	+	+
SMC-3	rod-shaped, light brown	−	−	+	+	+
SMC-4	rod-shaped, light brown	−	−	+	+	+
SMC-5	rod-shaped, light brown	−	−	+	+	+
FPA-1	rod-shaped, reddish brown	−	−	+	+	+
FPA-2	rod-shaped, reddish brown	−	−	+	+	+
FPA-3	rod-shaped, reddish brown	−	−	+	+	+
FPA-4	Coccus, white	+	−	−	−	+
JGV-1	rod-shaped, light brown	−	−	+	+	+
JGV-2	rod-shaped, light brown	−	−	+	+	+
FPP-1	Coccus, white	+	−	−	−	−
FPP-3	Coccus, white	+	−	−	−	−
FPP-4	Coccus, white	+	−	−	−	−
FPS-3	Coccus, white	+	−	−	−	−
FPS-4	rod-shaped, light brown	−	−	+	+	+

Table 1. *Cont.*

Isolate	Morphological		Biological			D-Mannitol Assimilation
	Colony Morphology	Gram Staining	Gluconic Acid Test	Biofilm Formation	Catalase Test	
ACJ-1	rod-shaped, light brown	−	−	+	+	+
ACJ-2	rod-shaped, light brown	−	−	+	+	+
MPV-1	rod-shaped, light brown	−	−	+	+	+
PVJ-1	rod-shaped, light brown	−	−	+	+	+
PVJ-4	rod-shaped, light brown	−	−	+	+	+
PVJ-5	rod-shaped, light brown	−	−	+	+	+
VVJ-1	rod-shaped, reddish brown	−	+	+	+	+
VVJ-2	rod-shaped, reddish brown	−	+	+	+	+
AVJ-3	rod-shaped, light brown	−	−	+	+	+
PEV-1	rod-shaped, light brown	−	−	+	+	+
PEV-4	rod-shaped, light brown	−	−	+	+	+
URV-1	rod-shaped, light brown	−	−	+	+	+
URV-2	rod-shaped, light brown	−	−	+	+	+
KS-1	rod-shaped, light brown	−	−	+	+	+
KS-2	rod-shaped, light brown	−	−	+	+	+
KS-3	rod-shaped, light brown	−	−	+	+	+
PS-1	rod-shaped, light brown	−	−	+	+	+
WS-1	rod-shaped, light brown	−	−	+	+	+

+: positive or activated, −: negative or inactive.

3.1.2. Identification of Isolated Strains

The results from the identification of 37 strains of acetic acid based on their 16S rDNA gene sequences are shown in Table 2. Sixteen strains in the genus *A. pasteurianus*, three strains in *A. orientalis*, one strain in *A. cibinongensis*, seven strains in *A. pomorum*, three strains in *A. ascendens*, and five strains in *A. malorum* were identified. Two strains of *Glu. oxydans* in the genus *Gluconobacter* were identified.

Table 2. Identification of acetic acid bacteria isolated from 22 samples.

Strains No.	Species	Identities (%)	Strain Distinction	Source
SVC-04	*Acetobacter pasteurianus* [1]	99.9	SFT-1	Vinegar, Sunchon University b3 115, Republic of Korea
SVC-12		99.7	SFT-2	
SVC-14		99.7	SFT-3	
SVC-22		99.9	SFT-4	
SVC-38		99.9	SFT-5	
SVC-49		99.9	SFT-6	
SVC-410		99.8	SFT-7	
SVC-54		99.5	SFT-8	
FPA-1	*Acetobacter orientalis*	99.8	SFT-9	*Prunus armeniaca* (surface), Suncheon-si, Jeollanam-do, Republic of Korea
FPA-2		99.9	SFT-10	
FPA-3		99.9	SFT-11	
FPS-4	*Acetobacter cibinongensis*	99.6	SFT-12	*Prunus salicina* (surface), Suncheon-si, Jeollanam-do, Republic of Korea
JGV-1	*Acetobacter pasteurianus* [1]	99.8	SFT-13	Vinegar (persimmon), Jangseong-gun, Jeollanam-do, Republic of Korea
JGV-2		99.6	SFT-14	
SMC-1	*Acetobacter pasteurianus* [1]	99.7	SFT-15	Maesil cheong, Sunchon University b3 115, Republic of Korea
SMC-2		99.5	SFT-16	
SMC-3		99.8	SFT-17	
SMC-4		99.6	SFT-18	
SMC-5		99.7	SFT-19	
ACJ-1	*Acetobacter pomorum*	99.4	SFT-20	Vinegar (*Ananas comosus*), Jangseong-gun, Jeollanam-do, Republic of Korea
ACJ-2		99.7	SFT-21	
MPV-1	*Acetobacter pomorum*	99.5	SFT-22	Vinegar (*Malus pumila*), Jangseong-gun, Jeollanam-do, Republic of Korea

Table 2. *Cont.*

Strains No.	Species	Identities (%)	Strain Distinction	Source
PVJ-1	*Acetobacter pomorum*	99.7	SFT-23	Vinegar (*persimmon*), Jangseong-gun, Jeollanam-do, Republic of Korea
PVJ-4	*Acetobacter pomorum*	99.7	SFT-24	
PVJ-5	*Acetobacter pasteurianus* [1]	99.8	SFT-25	
VVJ-1	*Gluconobacter oxydans* [1]	99.7	SFT-26	Vinegar (*Vitis vinifera L.*), Jangseong-gun, Jeollanam-do, Republic of Korea
VVJ-2		99.9	SFT-27	
AVJ-3	*Acetobacter pomorum*	99.5	SFT-28	Vinegar (*Aronia melanocarpa*), Jangseong-gun, Jeollanam-do, Republic of Korea
PEV-1	*Acetobacter ascendens* [1]	99.9	SFT-29	Vinegar (*Passiflora edulis)*, Jangseong-gun, Jeollanam-do, Republic of Korea
PEV-4		99.9	SFT-30	
URV-1	*Acetobacter ascendens* [1]	99.9	SFT-31	Vinegar (Brown rice), Namwon-si, Jeollabuk-do, Republic of Korea
URV-2	*Acetobacter pomorum*	99.7	SFT-32	
KS-1	*Acetobacter pomorum*	99.8	SFT-33	Kombucha (Masontops), North York, ON, Canada
KS-2		99.9	SFT-34	
KS-3		99.9	SFT-35	
PS-1	*Acetobacter pomorum*	99.8	SFT-36	Peach cheong, Sunchon University b3 115, Republic of Korea
WS-1	*Acetobacter pomorum*	99.9	SFT-37	Wine, Sunchon University b3 115, Republic of Korea

[1] List of 21 microorganisms approved by the Ministry of Food and Drug Safety for use as food ingredients.

In Korea, the use of acetic acid bacteria is mainly limited to acetic acid fermentation [6,7]. Of the 37 isolated strains, 21 strains are included on the list of relevant acetic acid bacteria. Therefore, the fermentation characteristics of 21 applicable strains and 10 strains with limited capacity for fermentation of acetic acid were compared for selection of the most suitable strains.

3.2. Fermentation Characteristics of Selected Strains

3.2.1. Titratable Acidity (TTA)

Changes in titratable acidity according to the incubation periods for acetic acid bacteria are shown in Table 3. A titratable acidity of 0.11% first showed an increasing trend ranging between 0.45 and 1.48%, with increasing incubation time on the third day. A titratable acidity of more than 1% was detected in six strains of *A. pasteurianus* (SFT-1, 6, 7, 16), *A. orientalis* SFT-10, and *A. ascendens* SFT-30).

The titratable acidity increased with increasing incubation time. However, variation in the range of increase was observed according to the strain. In agreement with the results of a previous study reported by Eom et al. [34], differences in changes in titratable acidity according to fermentation time were observed in four species of *A. pasteurianus*, even in the same species or genera of bacteria from the same source of isolation. The formation of organic acids, the metabolic products of acetic acid bacteria, can be assessed using the titratable acidity according to cultivation, which has been reported as an important indicator in the selection of acetic acid bacteria [35]. In addition, the findings of this study demonstrated the importance of acid resistance and ethanol tolerance as factors in the selection of acetic acid bacteria most suitable for use in symbiotic fermentation in the production of Kombucha.

Therefore, an experiment was conducted for the evaluation of the growth rate according to pH and alcohol concentration for a selection of exceptional strains.

Table 3. Comparative analysis of titratable acidity changes during the growth of select bacterial strains.

Sample	Titratable Acidity (%)			
	Fermentation Time (Days)			
	0	1	2	3
Acetobacter pasteurianus (SFT-1)	0.11 ± 0.00 [1,ns]	0.27 ± 0.00 [b]	0.64 ± 0.03 [d]	1.18 ± 0.00 [d]
Acetobacter pasteurianus (SFT-2)		0.22 ± 0.00 [ef]	0.42 ± 0.02 [hij]	0.75 ± 0.00 [hi]
Acetobacter pasteurianus (SFT-3)		0.24 ± 0.00 [c]	0.49 ± 0.00 [f]	0.87 ± 0.01 [f]
Acetobacter pasteurianus (SFT-4)		0.19 ± 0.01 [hi]	0.36 ± 0.00 [lmno]	0.55 ± 0.00 [no]
Acetobacter pasteurianus (SFT-5)		0.25 ± 0.01 [c]	0.45 ± 0.05 [gh]	0.85 ± 0.03 [f]
Acetobacter pasteurianus (SFT-6)		0.21 ± 0.01 [fg]	0.60 ± 0.01 [e]	1.07 ± 0.05 [e]
Acetobacter pasteurianus (SFT-7)		0.25 ± 0.00 [c]	0.73 ± 0.01 [b]	1.39 ± 0.01 [b]
Acetobacter pasteurianus (SFT-8)		0.23 ± 0.01 [de]	0.48 ± 0.02 [fg]	0.82 ± 0.03 [fg]
Acetobacter orientalis (SFT-10)		0.28 ± 0.02 [ab]	0.84 ± 0.02 [a]	1.48 ± 0.05 [a]
Acetobacter cibinongensis (SFT-12)		0.21 ± 0.00 [fg]	0.34 ± 0.01 [nop]	0.60 ± 0.02 [lmn]
Acetobacter pasteurianus (SFT-13)		0.21 ± 0.01 [fg]	0.29 ± 0.00 [opq]	0.45 ± 0.03 [q]
Acetobacter pasteurianus (SFT-14)		0.21 ± 0.00 [fg]	0.28 ± 0.01 [r]	0.38 ± 0.01 [r]
Acetobacter pasteurianus (SFT-15)		0.13 ± 0.01 [k]	0.32 ± 0.01 [pq]	0.70 ± 0.03 [ijk]
Acetobacter pasteurianus (SFT-16)		0.29 ± 0.02 [a]	0.70 ± 0.02 [bc]	1.28 ± 0.02 [c]
Acetobacter pasteurianus (SFT-17)		0.24 ± 0.02 [c]	0.37 ± 0.02 [lmn]	0.59 ± 0.06 [mno]
Acetobacter pasteurianus (SFT-18)		0.14 ± 0.00 [jk]	0.27 ± 0.02 [r]	0.72 ± 0.06 [ij]
Acetobacter pasteurianus (SFT-19)		0.22 ± 0.00 [ef]	0.36 ± 0.01 [lmno]	0.57 ± 0.01 [mno]
Acetobacter pomorum (SFT-21)		0.18 ± 0.00 [i]	0.30 ± 0.00 [qr]	0.54 ± 0.01 [op]
Acetobacter pomorum (SFT-22)		0.21 ± 0.00 [fg]	0.34 ± 0.00 [nop]	0.54 ± 0.03 [op]
Acetobacter pomorum (SFT-24)		0.22 ± 0.00 [ef]	0.43 ± 0.02 [hi]	0.75 ± 0.06 [hi]
Acetobacter pasteurianus (SFT-25)		0.20 ± 0.00 [gh]	0.38 ± 0.01 [klm]	0.68 ± 0.03 [jk]
Gluconobacter oxydans (SFT-26)		0.15 ± 0.01 [j]	0.20 ± 0.02 [s]	0.45 ± 0.01 [q]
Gluconobacter oxydans (SFT-27)		0.14 ± 0.00 [jk]	0.21 ± 0.00 [s]	0.47 ± 0.02 [q]
Acetobacter pomorum (SFT-28)		0.22 ± 0.00 [ef]	0.39 ± 0.01 [jkl]	0.65 ± 0.01 [kl]
Acetobacter ascendens (SFT-29)		0.23 ± 0.01 [de]	0.41 ± 0.01 [ijk]	0.73 ± 0.01 [hij]
Acetobacter ascendens (SFT-30)		0.27 ± 0.01 [b]	0.69 ± 0.01 [c]	1.36 ± 0.01 [b]
Acetobacter ascendens (SFT-31)		0.13 ± 0.01 [k]	0.35 ± 0.02 [mnop]	0.62 ± 0.01 [lm]
Acetobacter pomorum (SFT-32)		0.14 ± 0.00 [jk]	0.37 ± 0.01 [lmn]	0.70 ± 0.01 [ijk]
Acetobacter malorum (SFT-33)		0.25 ± 0.01 [c]	0.43 ± 0.03 [hi]	0.69 ± 0.01 [jk]
Acetobacter malorum (SFT-36)		0.27 ± 0.00 [b]	0.51 ± 0.00 [f]	0.78 ± 0.04 [gh]
Acetobacter malorum (SFT-37)		0.24 ± 0.01 [cde]	0.33 ± 0.00 [opq]	0.49 ± 0.04 [pq]

[1] All values are mean ± SD (n = 3).; ns, non-significance.; Means with different superscript letters in the same column are significantly different at $p < 0.05$ by Duncan's multiple range test. a > b > c > d > e > f > g > h > I > j > k > l > m > n > o > p > q > r > s.

3.2.2. Acid Resistance

Microbial growth, including that of acetic acid bacteria, is inhibited in an acidic environment; thus, the selection of acid-resistant strains is a critical factor. The results regarding the acid resistance of strains according to the pH concentrations of the culture solution are shown in Table 4. Variation in the resistance confirmed by growth, according to acid concentration, was observed for each strain. However, overall, low growth was observed at pH 8.0 and high growth at pH 5.0 to 7.0. Bang et al. [36] suggested a pH range of 5.5–6.5 as an optimum condition for the growth of acetic acid bacteria. Park et al. [37] reported favorable outcomes for microbial growth at pH 4.0–6.0. These previously reported findings are comparable to the results obtained in this study. Variation in growth rates in the same pH range was observed even in the same genera and species of bacterial strains. The following strains showed a growth rate of 100% or higher in the pH range 4.0–5.0: *A. pasteurianus* SFT-3, 4, 7, 13, 16, 18, and 19; *A. pomorum* SFT-24, 28, and 32; *A. ascendens* SFT-31; and *A. malorum* SFT-36 and 37. *Glu. oxydans* SFT-26 and 27 showed increased growth rates at higher pH, which rose to 144.16% and 251.50% at an optimum growth pH of 6.0. Gupta et al. [32] recommended an optimum pH of 5.5–6.5 to support growth in all strains of *Gluconobacter*, comparable to the outcome of this study.

Table 4. Evaluation of growth rate of acetic acid bacteria according to pH and alcohol concentration.

Sample \ Treatment	Acid Resistance (%)					Ethanol Tolerance (%)				
	pH					Alcohol Content (%)				
	4.0	5.0	6.0	7.0	8.0	2.0	4.0	6.0	8.0	10.0
Acetobacter pasteurianus (SFT-1)	90.80 ± 0.08 [1,d]	112.89 ± 0.16 [a]	98.33 ± 0.09 [c]	100.13 ± 0.09 [b]	84.26 ± 0.06 [e]	95.64 ± 0.07 [a]	86.92 ± 0.14 [e]	94.45 ± 0.07 [b]	88.45 ± 0.21 [c]	87.97 ± 0.07 [d]
Acetobacter pasteurianus (SFT-2)	80.73 ± 0.09 [c]	99.60 ± 0.08 [b]	73.57 ± 0.03 [d]	101.99 ± 0.00 [a]	61.46 ± 0.09 [e]	108.78 ± 0.23 [d]	126.33 ± 0.46 [b]	129.33 ± 0.12 [a]	123.21 ± 0.23 [c]	122.75 ± 0.46 [c]
Acetobacter pasteurianus (SFT-3)	119.84 ± 0.23 [b]	132.10 ± 0.11 [a]	91.19 ± 0.11 [e]	100.66 ± 0.11 [c]	97.10 ± 0.12 [d]	92.76 ± 0.07 [a]	78.27 ± 0.00 [d]	76.56 ± 0.32 [e]	80.61 ± 0.26 [c]	81.37 ± 0.19 [b]
Acetobacter pasteurianus (SFT-4)	100.63 ± 0.19 [b]	118.18 ± 0.019 [a]	95.43 ± 0.10 [c]	100.34 ± 0.29 [b]	87.13 ± 0.29 [d]	97.09 ± 0.08 [a]	91.26 ± 0.14 [b]	80.31 ± 0.22 [c]	77.11 ± 0.08 [d]	67.52 ± 0.36 [e]
Acetobacter pasteurianus (SFT-5)	97.54 ± 0.10 [c]	128.84 ± 0.10 [a]	96.46 ± 0.20 [d]	100.27 ± 0.10 [b]	82.12 ± 0.10 [e]	94.22 ± 0.13 [a]	82.66 ± 0.26 [b]	77.04 ± 0.13 [c]	74.99 ± 0.26 [d]	71.16 ± 0.06 [e]
Acetobacter pasteurianus (SFT-6)	66.05 ± 0.16 [e]	72.01 ± 0.16 [d]	92.97 ± 0.16 [b]	100.51 ± 0.16 [a]	85.35 ± 0.24 [c]	94.01 ± 0.00 [a]	82.02 ± 0.20 [b]	77.98 ± 0.07 [c]	72.26 ± 0.21 [d]	67.61 ± 0.27 [e]
Acetobacter pasteurianus (SFT-7)	111.67 ± 0.12 [b]	136.56 ± 0.45 [a]	102.82 ± 0.23 [c]	99.79 ± 0.00 [d]	81.51 ± 0.11 [e]	88.07 ± 0.24 [a]	64.22 ± 0.12 [e]	80.16 ± 0.18 [c]	74.17 ± 0.06 [d]	83.07 ± 0.12 [b]
Acetobacter pasteurianus (SFT-8)	120.77 ± 0.32 [a]	92.80 ± 0.11 [d]	75.96 ± 0.11 [e]	101.81 ± 0.11 [b]	100.75 ± 0.21 [a]	100.05 ± 0.28 [a]	100.14 ± 0.09 [a]	88.99 ± 0.18 [d]	91.07 ± 0.46 [b]	90.35 ± 0.09 [c]
Acetobacter orientalis (SFT-10)	111.49 ± 0.14 [b]	96.01 ± 0.14 [d]	89.90 ± 0.41 [e]	100.76 ± 0.27 [c]	114.75 ± 0.68 [a]	97.64 ± 0.12 [b]	92.92 ± 0.37 [c]	79.25 ± 0.25 [e]	85.71 ± 0.63 [d]	169.44 ± 0.10 [a]
Acetobacter cibinongensis (SFT-12)	56.19 ± 0.25 [d]	32.96 ± 0.25 [e]	97.43 ± 0.00 [b]	100.19 ± 0.33 [a]	78.19 ± 0.08 [c]	119.63 ± 0.33 [c]	158.89 ± 0.11 [a]	158.24 ± 0.33 [a]	132.97 ± 0.65 [b]	114.32 ± 0.22 [d]
Acetobacter pasteurianus (SFT-13)	115.76 ± 0.11 [b]	144.67 ± 0.11 [a]	107.28 ± 0.11 [c]	99.45 ± 0.22 [d]	98.80 ± 0.11 [e]	99.37 ± 0.08 [c]	98.11 ± 0.34 [d]	96.94 ± 0.25 [e]	102.89 ± 0.09 [b]	106.33 ± 0.09 [a]
Acetobacter pasteurianus (SFT-14)	90.93 ± 0.09 [c]	103.69 ± 0.34 [a]	103.69 ± 0.17 [a]	99.72 ± 0.09 [b]	78.17 ± 0.17 [d]	100.84 ± 0.09 [d]	102.52 ± 0.28 [c]	106.26 ± 0.19 [b]	118.21 ± 0.10 [a]	102.71 ± 0.47 [c]
Acetobacter pasteurianus (SFT-15)	92.04 ± 0.23 [c]	107.68 ± 0.08 [a]	82.95 ± 0.39 [d]	101.28 ± 0.23 [b]	81.41 ± 0.00 [e]	103.47 ± 0.15 [b]	110.41 ± 0.15 [a]	100.27 ± 0.31 [d]	102.10 ± 0.31 [c]	96.30 ± 0.08 [e]
Acetobacter pasteurianus (SFT-16)	108.33 ± 0.10 [b]	117.40 ± 0.30 [a]	101.78 ± 0.10 [c]	99.87 ± 0.10 [d]	80.62 ± 0.10 [e]	86.79 ± 0.05 [a]	60.37 ± 0.11 [b]	60.48 ± 0.22 [b]	59.94 ± 0.06 [c]	55.82 ± 0.22 [d]
Acetobacter pasteurianus (SFT-17)	98.79 ± 0.21 [d]	130.46 ± 0.21 [a]	94.28 ± 0.10 [e]	100.43 ± 0.10 [c]	124.21 ± 0.21 [b]	101.76 ± 0.08 [c]	105.27 ± 0.24 [a]	91.37 ± 0.08 [e]	93.53 ± 0.24 [d]	102.56 ± 0.08 [b]
Acetobacter pasteurianus (SFT-18)	114.56 ± 0.11 [b]	120.88 ± 0.11 [a]	97.88 ± 0.21 [d]	100.16 ± 0.00 [c]	86.07 ± 0.11 [e]	94.82 ± 0.07 [a]	84.46 ± 0.27 [c]	79.41 ± 0.07 [d]	92.16 ± 0.14 [b]	55.76 ± 0.07 [e]
Acetobacter pasteurianus (SFT-19)	108.94 ± 0.10 [b]	119.27 ± 0.20 [a]	100.18 ± 0.10 [c]	99.99 ± 0.20 [c]	83.85 ± 0.59 [d]	93.66 ± 0.13 [a]	80.98 ± 0.06 [b]	74.14 ± 0.13 [c]	72.00 ± 0.00 [d]	66.60 ± 0.07 [e]
Acetobacter pomorum (SFT-21)	100.31 ± 0.26 [a]	99.29 ± 0.09 [b]	93.59 ± 0.09 [c]	100.48 ± 0.09 [a]	89.84 ± 0.09 [d]	92.57 ± 0.17 [a]	77.72 ± 0.25 [b]	76.67 ± 0.09 [c]	71.83 ± 0.32 [d]	68.04 ± 0.08 [e]
Acetobacter pomorum (SFT-22)	93.01 ± 0.21 [d]	120.86 ± 0.07 [a]	91.66 ± 0.14 [e]	100.63 ± 0.14 [b]	93.68 ± 0.27 [c]	94.96 ± 0.06 [a]	84.87 ± 0.11 [b]	83.01 ± 0.06 [c]	61.26 ± 0.12 [d]	55.28 ± 0.23 [e]
Acetobacter pomorum (SFT-24)	102.28 ± 0.07 [c]	114.51 ± 0.07 [b]	123.88 ± 0.50 [a]	98.20 ± 0.22 [d]	80.68 ± 0.22 [e]	95.70 ± 0.13 [a]	87.10 ± 0.13 [c]	87.42 ± 0.07 [b]	57.80 ± 0.07 [e]	65.39 ± 0.00 [d]
Acetobacter pasteurianus (SFT-25)	84.52 ± 0.07 [e]	94.49 ± 0.15 [b]	90.86 ± 0.43 [c]	100.69 ± 0.07 [a]	89.94 ± 0.08 [d]	97.43 ± 0.46 [a]	92.29 ± 0.08 [d]	86.77 ± 0.08 [e]	96.43 ± 0.54 [b]	93.44 ± 0.23 [c]
Gluconobacter oxydans (SFT-26)	43.82 ± 0.23 [e]	60.18 ± 0.11 [d]	144.16 ± 0.23 [b]	96.68 ± 0.12 [c]	185.12 ± 0.35 [a]	137.11 ± 0.19 [c]	211.32 ± 0.19 [a]	94.81 ± 0.56 [e]	130.98 ± 0.19 [d]	172.73 ± 0.56 [b]
Gluconobacter oxydans (SFT-27)	72.19 ± 0.13 [e]	86.73 ± 0.25 [d]	251.50 ± 0.37 [a]	88.60 ± 0.25 [c]	171.23 ± 0.25 [b]	96.48 ± 0.12 [d]	89.44 ± 0.24 [e]	143.57 ± 0.00 [c]	158.50 ± 0.25 [a]	148.18 ± 0.36 [b]
Acetobacter pomorum (SFT-28)	109.16 ± 0.25 [b]	102.64 ± 0.25 [c]	116.01 ± 0.09 [a]	98.79 ± 0.17 [d]	93.19 ± 0.42 [e]	103.35 ± 0.07 [b]	110.05 ± 0.15 [a]	101.33 ± 0.29 [c]	69.72 ± 0.08 [d]	66.33 ± 0.00 [e]
Acetobacter ascendens (SFT-29)	92.54 ± 0.41 [e]	99.01 ± 0.08 [c]	94.89 ± 0.08 [d]	100.38 ± 0.09 [b]	105.23 ± 0.09 [a]	94.46 ± 0.15 [a]	83.37 ± 0.07 [b]	77.61 ± 0.15 [d]	78.79 ± 0.08 [c]	69.70 ± 0.22 [e]
Acetobacter ascendens (SFT-30)	95.06 ± 0.11 [e]	101.59 ± 0.21 [c]	106.45 ± 0.31 [a]	99.51 ± 0.11 [d]	103.97 ± 0.42 [b]	96.04 ± 0.08 [a]	88.12 ± 0.16 [b]	74.21 ± 0.08 [d]	77.33 ± 0.24 [c]	70.37 ± 0.08 [e]
Acetobacter ascendens (SFT-31)	121.31 ± 0.25 [c]	154.97 ± 0.12 [a]	125.67 ± 0.12 [b]	98.07 ± 0.24 [d]	90.80 ± 0.49 [e]	87.24 ± 0.09 [c]	61.72 ± 0.19 [e]	89.13 ± 0.28 [b]	107.37 ± 0.10 [a]	78.36 ± 0.10 [d]
Acetobacter pomorum (SFT-32)	156.12 ± 0.13 [a]	122.40 ± 0.13 [b]	102.64 ± 0.25 [c]	99.80 ± 0.12 [d]	93.75 ± 0.12 [e]	153.29 ± 1.20 [d]	259.88 ± 0.24 [a]	212.69 ± 1.68 [b]	139.64 ± 0.72 [e]	159.28 ± 0.24 [c]
Acetobacter malorum (SFT-33)	117.70 ± 0.08 [a]	89.90 ± 0.29 [d]	75.35 ± 0.07 [e]	101.86 ± 0.22 [c]	117.34 ± 0.15 [b]	72.20 ± 0.16 [a]	16.59 ± 0.07 [e]	23.63 ± 0.00 [d]	43.03 ± 0.07 [b]	29.98 ± 0.04 [c]
Acetobacter malorum (SFT-36)	176.67 ± 0.25 [a]	125.85 ± 0.08 [b]	82.67 ± 0.25 [d]	101.30 ± 0.09 [c]	55.66 ± 0.49 [e]	80.09 ± 0.07 [a]	40.28 ± 0.23 [b]	23.66 ± 0.07 [c]	17.47 ± 0.03 [e]	19.66 ± 0.20 [d]
Acetobacter malorum (SFT-37)	100.68 ± 0.07 [c]	118.63 ± 0.07 [a]	105.50 ± 0.08 [b]	99.59 ± 0.44 [d]	64.57 ± 0.15 [e]	78.76 ± 0.04 [a]	36.27 ± 0.08 [b]	19.86 ± 0.12 [d]	30.14 ± 0.08 [c]	36.3 ± 0.12 [b]

[1] All values are mean ± SD (n = 3); Means with different superscript letters in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test. a > b > c > d > e.

3.2.3. Alcohol Tolerance

Sugars are converted into alcohol by yeast and ethanol is oxidized into acetic acid by acetic acid bacteria during the process of symbiotic fermentation; thus, alcohol concentration is an important factor in microbial growth and acid production [38]. However, a high concentration of ethanol during the initial period can result in a delay of the induction period, leading to deceleration of the growth of acetic acid bacteria along with a reduction in acid productivity [39]. The growth rates of acetic acid bacteria according to ethanol concentrations are shown in Table 4. Most isolated strains of acetic acid bacteria showed reduced growth rates at a concentration of 10% ethanol. This result is consistent with those of an earlier study, which reported lower growth of acetic acid bacteria at an ethanol content of 9% [36]. An increase in growth rates to higher than 100% was observed at ethanol concentrations of 8–10% in *A. pasteurianus* SFT-2, 13, 14, and 17; *A. orientalis* SFT-10; *A. cibinongensis* SFT-12; *A. pomorum* SFT-32; and *Glu. oxydans* SFT-26 and 27, indicating high alcohol tolerance.

An optimum alcohol concentration of 4% for acetic acid fermentation has been reported [40]. According to an earlier study on capacity in the production of acetic acid, the activity of acetic acid production was affected by the characteristics of bacterial strains [41]. Therefore, additional studies are warranted in order to further determine the capacity in the production of acetic acid according to characteristics of the strain. In the selection of strains, this study complied with regulations for food standards and specifications established by the Korean Ministry of Food and Drug Safety for microorganisms approved for use as food materials [5], and assessment of factors impeding the growth and formation of bacteria used in Kombucha fermentation was performed [42–44].

The screening of acetic acid bacteria suitable for use in symbiotic fermentation, including five strains of the genus *Acetobacter* (*A. pasteurianus* SFT-3, 13, 18, and *A. ascendens* SFT-30, 31) and two strains of the genus *Gluconobacter* (*Glu. oxydans* SFT-26, 27) was based on the evaluation of fermentation characteristics (pH and titratable acidity), acid resistance, and alcohol tolerance. An impact evaluation according to fermentation temperature and fermentation period was conducted using the seven selected strains.

3.3. Fermentation Characteristics According to Fermentation Temperature and Time

3.3.1. pH, Titratable Acidity, and Viable Cell Count

The results regarding the optimum temperature for the growth of acetic acid bacteria and determining the incubation period are shown in Table 5. Changes in the overall pH showed a severe decrease from the initial pH between day 0 and day 2, and the lowest pH values were detected in *A. pasteurianus* SFT-18 (pH 3.85) and *Glu. oxydans* SFT-27 (pH 3.56) on the day 3. According to incubation temperature, a large-scale decrease in pH was observed at a temperature range of 30–35 °C. The pH values were reduced by 2.46, compared to the initial pH at an incubation temperature of 35 °C in *A. pasteurianus* SFT-18, and by 2.90 at 30 °C in *Glu. oxydans* SFT-26.

Most strains showed a gradual decrease in titratable acidity. The highest titratable acidity was detected in *A. pasteurianus* SFT-18 (1.68%) and *Glu. oxydans* SFT-27 (0.79%) on the third day. According to incubation temperature, the titratable acidity showed a substantial increase at 30–35 °C. The titratable acidity increased by 1.56 compared with the initial titratable acidity observed at an incubation temperature of 35 °C in *A. pasteurianus* SFT-18 and by 0.67 at 35 °C in *Glu. oxydans* SFT-27.

Table 5. Fermentation characteristics of initially selected acetic acid bacteria strains based on fermentation temperature and period.

Sample		pH				Titratable Acidity (%)				Microbial Count (logCFU/mL)			
	Day	25 °C	30 °C	35 °C	40 °C	25 °C	30 °C	35 °C	40 °C	25 °C	30 °C	35 °C	40 °C
Acetobacter pasteurianus (SFT-3)	0	6.14 ± 0.01 [1,ns]	6.14 ± 0.00	6.12 ± 0.01	6.13 ± 0.02	0.10 ± 0.01 [bc]	0.12 ± 0.00 [a]	0.11 ± 0.01 [ab]	0.09 ± 0.01 [c]	6.13 ± 0.08 [ns]	6.08 ± 0.15	6.22 ± 0.07	6.22 ± 0.07
	1	5.18 ± 0.01 [b]	4.94 ± 0.01 [c]	4.61 ± 0.00 [d]	5.67 ± 0.00 [a]	0.14 ± 0.01 [c]	0.21 ± 0.00 [b]	0.34 ± 0.03 [a]	0.10 ± 0.02 [d]	6.40 ± 0.52 [ns]	6.36 ± 0.67	6.53 ± 0.25	6.24 ± 0.49
	2	4.61 ± 0.01 [b]	4.60 ± 0.01 [b]	4.22 ± 0.01 [c]	5.07 ± 0.00 [a]	0.35 ± 0.01 [b]	0.38 ± 0.03 [b]	0.81 ± 0.03 [a]	0.18 ± 0.00 [c]	7.15 ± 0.45 [b]	7.17 ± 0.42 [b]	8.05 ± 0.67 [a]	6.95 ± 0.07 [b]
	3	4.35 ± 0.01 [b]	4.32 ± 0.00 [c]	3.97 ± 0.01 [d]	4.50 ± 0.01 [a]	0.57 ± 0.00 [b]	0.60 ± 0.03 [b]	1.32 ± 0.02 [a]	0.41 ± 0.01 [c]	8.81 ± 0.95 [ns]	8.85 ± 0.77	8.92 ± 0.98	8.64 ± 0.81
Acetobacter pasteurianus (SFT-13)	0	6.42 ± 0.00 [a]	6.40 ± 0.01 [b]	6.38 ± 0.01 [c]	6.41 ± 0.00 [ab]	0.09 ± 0.00 [b]	0.10 ± 0.02 [ab]	0.12 ± 0.01 [a]	0.09 ± 0.01 [b]	6.36 ± 0.08 [c]	6.54 ± 0.04 [b]	6.71 ± 0.02 [a]	6.53 ± 0.03 [b]
	1	5.32 ± 0.01 [a]	4.92 ± 0.00 [b]	4.84 ± 0.01 [c]	4.68 ± 0.00 [d]	0.13 ± 0.01 [d]	0.21 ± 0.01 [c]	0.24 ± 0.01 [b]	0.31 ± 0.01 [a]	6.46 ± 0.34 [c]	6.77 ± 0.19 [bc]	6.92 ± 0.19 [b]	7.42 ± 0.12 [a]
	2	4.78 ± 0.01 [a]	4.50 ± 0.00 [b]	4.46 ± 0.01 [c]	4.26 ± 0.00 [d]	0.25 ± 0.01 [d]	0.44 ± 0.00 [c]	0.47 ± 0.02 [b]	0.77 ± 0.02 [a]	7.54 ± 0.34 [ns]	8.18 ± 0.57	8.41 ± 0.66	8.58 ± 0.57
	3	4.50 ± 0.00 [a]	4.24 ± 0.00 [b]	4.11 ± 0.00 [c]	4.00 ± 0.01 [d]	0.42 ± 0.00 [d]	0.71 ± 0.02 [c]	0.92 ± 0.01 [b]	1.29 ± 0.01 [a]	8.70 ± 0.71 [ns]	9.00 ± 0.75	9.05 ± 0.82	8.99 ± 0.86
Acetobacter pasteurianus (SFT-18)	0	6.34 ± 0.00 [b]	6.34 ± 0.01 [b]	6.31 ± 0.02 [c]	6.37 ± 0.01 [a]	0.10 ± 0.01 [b]	0.09 ± 0.01 [b]	0.12 ± 0.01 [a]	0.09 ± 0.00 [b]	5.93 ± 0.13 [ns]	5.93 ± 0.11	5.89 ± 0.10	6.01 ± 0.12
	1	4.89 ± 0.01 [a]	4.87 ± 0.01 [b]	4.49 ± 0.01 [c]	4.89 ± 0.01 [a]	0.22 ± 0.01 [b]	0.22 ± 0.01 [b]	0.43 ± 0.02 [a]	0.22 ± 0.01 [b]	6.67 ± 0.02 [bc]	6.75 ± 0.02 [b]	6.98 ± 0.18 [a]	6.54 ± 0.10 [c]
	2	4.24 ± 0.01 [c]	4.43 ± 0.00 [b]	4.12 ± 0.01 [d]	4.54 ± 0.00 [a]	0.75 ± 0.01 [b]	0.53 ± 0.02 [c]	0.98 ± 0.01 [a]	0.43 ± 0.00 [d]	8.11 ± 0.64 [ns]	7.76 ± 0.35	8.40 ± 0.50	7.43 ± 0.60
	3	3.93 ± 0.00 [c]	4.10 ± 0.01 [b]	3.85 ± 0.00 [d]	4.31 ± 0.01 [a]	1.35 ± 0.00 [b]	0.95 ± 0.02 [c]	1.68 ± 0.02 [a]	0.66 ± 0.01 [d]	9.42 ± 0.81 [ns]	9.23 ± 0.76	9.52 ± 0.49	9.01 ± 0.72
Acetobacter ascendens (SFT-30)	0	6.33 ± 0.01 [ab]	6.31 ± 0.02 [b]	6.33 ± 0.01 [ab]	6.34 ± 0.01 [a]	0.09 ± 0.02 [ns]	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	6.50 ± 0.03 [c]	6.77 ± 0.04 [a]	6.65 ± 0.02 [b]	6.46 ± 0.06 [c]
	1	5.08 ± 0.00 [b]	4.89 ± 0.02 [d]	4.99 ± 0.00 [c]	5.30 ± 0.01 [a]	0.16 ± 0.02 [b]	0.22 ± 0.01 [a]	0.20 ± 0.01 [a]	0.13 ± 0.01 [c]	7.10 ± 0.23 [b]	7.77 ± 0.02 [a]	6.83 ± 0.07 [c]	6.68 ± 0.15 [c]
	2	4.59 ± 0.00 [c]	4.52 ± 0.00 [d]	4.72 ± 0.01 [b]	4.87 ± 0.00 [a]	0.37 ± 0.01 [b]	0.42 ± 0.02 [a]	0.31 ± 0.01 [c]	0.24 ± 0.01 [d]	8.05 ± 0.89 [ns]	8.51 ± 0.58 [b]	7.51 ± 0.68	7.34 ± 0.58
	3	4.31 ± 0.01 [c]	4.26 ± 0.01 [d]	4.49 ± 0.01 [b]	4.55 ± 0.01 [a]	0.63 ± 0.01 [b]	0.68 ± 0.00 [a]	0.44 ± 0.02 [c]	0.41 ± 0.01 [d]	9.10 ± 0.95 [ns]	9.30 ± 1.19	8.99 ± 0.73	8.91 ± 0.66
Acetobacter ascendens (SFT-31)	0	6.40 ± 0.01 [ab]	6.38 ± 0.02 [bc]	6.37 ± 0.01 [c]	6.41 ± 0.01 [a]	0.08 ± 0.00 [b]	0.11 ± 0.00 [a]	0.12 ± 0.01 [a]	0.08 ± 0.01 [b]	6.36 ± 0.06 [b]	6.48 ± 0.06 [ab]	6.55 ± 0.09 [a]	6.37 ± 0.06 [b]
	1	5.75 ± 0.00 [b]	5.30 ± 0.01 [c]	4.88 ± 0.00 [d]	5.89 ± 0.01 [a]	0.11 ± 0.02 [c]	0.15 ± 0.00 [b]	0.21 ± 0.01 [a]	0.09 ± 0.00 [c]	6.82 ± 0.15 [bc]	7.12 ± 0.08 [ab]	7.41 ± 0.18 [a]	6.39 ± 0.53 [c]
	2	5.04 ± 0.01 [b]	4.65 ± 0.00 [c]	4.57 ± 0.01 [d]	5.71 ± 0.00 [a]	0.19 ± 0.02 [b]	0.38 ± 0.02 [a]	0.39 ± 0.01 [a]	0.12 ± 0.02 [c]	7.52 ± 0.60 [ns]	8.19 ± 0.50	8.40 ± 0.63	7.36 ± 0.51
	3	4.37 ± 0.01 [b]	4.35 ± 0.01 [c]	4.34 ± 0.01 [c]	5.46 ± 0.01 [a]	0.58 ± 0.02 [b]	0.61 ± 0.00 [a]	0.61 ± 0.02 [a]	0.14 ± 0.00 [c]	8.37 ± 0.81 [ns]	8.95 ± 0.75	8.90 ± 0.94	8.04 ± 0.58
Gluconobacter oxydans (SFT-26)	0	6.68 ± 0.01 [a]	6.67 ± 0.00 [a]	6.64 ± 0.01 [b]	6.67 ± 0.00 [a]	0.09 ± 0.01 [ns]	0.09 ± 0.01	0.10 ± 0.00	0.09 ± 0.01	6.11 ± 0.07 [b]	6.20 ± 0.01 [a]	6.27 ± 0.02 [a]	6.23 ± 0.02 [a]
	1	5.06 ± 0.00 [d]	5.24 ± 0.02 [c]	5.76 ± 0.01 [b]	6.12 ± 0.01 [a]	0.13 ± 0.01 [ns]	0.12 ± 0.02	0.11 ± 0.02	0.10 ± 0.01	7.51 ± 0.21 [a]	6.94 ± 0.17 [ab]	6.45 ± 0.38 [b]	6.35 ± 0.76 [b]
	2	4.31 ± 0.01 [d]	4.33 ± 0.01 [c]	4.65 ± 0.00 [b]	6.02 ± 0.00 [a]	0.24 ± 0.02 [a]	0.24 ± 0.02 [a]	0.18 ± 0.00 [b]	0.10 ± 0.00 [c]	8.37 ± 0.79 [ns]	8.35 ± 0.83	8.09 ± 0.84	7.34 ± 0.58
	3	3.80 ± 0.01 [c]	3.77 ± 0.00 [d]	4.46 ± 0.00 [b]	5.89 ± 0.00 [a]	0.52 ± 0.02 [a]	0.52 ± 0.00 [a]	0.21 ± 0.00 [b]	0.11 ± 0.00 [c]	9.54 ± 0.68 [ns]	9.41 ± 0.53	9.37 ± 0.53	8.48 ± 0.63
Gluconobacter oxydans (SFT-27)	0	6.22 ± 0.01 [a]	6.21 ± 0.00 [ab]	6.20 ± 0.00 [b]	6.21 ± 0.01 [ab]	0.11 ± 0.00 [ns]	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.00	5.87 ± 0.46 [ns]	5.97 ± 0.69	6.21 ± 0.30	6.11 ± 0.35
	1	5.57 ± 0.01 [b]	5.12 ± 0.01 [d]	5.30 ± 0.00 [c]	5.88 ± 0.00 [a]	0.13 ± 0.01 [a]	0.14 ± 0.01 [a]	0.13 ± 0.00 [a]	0.11 ± 0.01 [b]	7.49 ± 0.29 [ns]	7.28 ± 0.43	7.00 ± 0.47	6.89 ± 0.90
	2	4.68 ± 0.01 [b]	3.84 ± 0.01 [d]	3.90 ± 0.01 [c]	5.83 ± 0.02 [a]	0.16 ± 0.01 [c]	0.45 ± 0.01 [a]	0.38 ± 0.00 [b]	0.11 ± 0.00 [d]	7.86 ± 0.36 [ns]	8.01 ± 0.60	8.04 ± 0.70	7.41 ± 0.61
	3	3.63 ± 0.00 [b]	3.56 ± 0.02 [c]	3.55 ± 0.00 [c]	5.83 ± 0.01 [a]	0.73 ± 0.00 [c]	0.75 ± 0.02 [b]	0.79 ± 0.00 [a]	0.13 ± 0.00 [d]	8.19 ± 0.71 [ns]	8.44 ± 0.87	8.55 ± 0.76	8.09 ± 0.77

[1] All values are mean ± SD (n = 3); ns, non-significance; Means with different superscript letters in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test. a > b > c > d.

Regarding changes in viable cell count according to fermentation temperature, all strains showed high growth rates at a temperature of 35 °C or below. High numbers of viable cells were detected according to temperature in *A. pasteurianus* SFT-3 (8.92 logCFU/mL), *A. pasteurianus* SFT-13 (9.05 logCFU/mL), *A. pasteurianus* SFT-18 (9.52 logCFU/mL), and *Glu. oxydans* SFT-27 (8.55 logCFU/mL) at 35 °C; *A. ascendens* SFT-30 (9.30 logCFU/mL) and *A. ascendens* SFT-31 (8.95 logCFU/mL) at 30 °C; and *Glu. oxydans* SFT-26 (9.54 logCFU/mL) at 25 °C. In particular, a wider range of incubation temperatures was observed for growth of *A. pasteurianus* SFT-18 compared with other bacterial strains, with increases in the number of viable cells to 3.49 logCFU/mL at 25 °C, 3.30 logCFU/mL at 30 °C, 3.63 logCFU/mL at 35 °C, and 3.00 logCFU/mL at 40 °C from the initial viable cell count.

Despite variation in acid productivity according to the isolated strain, a significant change in pH and total acidity content was observed as the viable cell count increased in the same strain. Despite an increase in the viable cell count to 1.67–2.25 logCFU/mL in *A. ascendens* SFT-31 and *Glu. oxydans* SFT-26 and 27 at 40 °C compared to the day 0, the change in titratable acidity and pH with the effect of metabolic products was insignificant. According to previous studies reported by Gullo et al. [45] and Sharafi et al. [46], inactivation of acetic acid bacteria may be a result of an irregularity in optimum growth temperature, resulting in a reduction in metabolism caused by injury to membranes. It is believed that these previous findings support the findings of the current study. Therefore, based on its stable fermentation characteristics at a wide range of culture temperatures, *A. pasteurianus* SFT-18 (Accession; CP015168.1, Description; *A. pasteurianus*, Length; 2810721, Start; 874219, End; 875683, Coverage; 0, Bit; 2673, E-Value; 0.0, Match/Total; 1460/1466, Pct. (%); 99.6) can be regarded as the most suitable strain for use in the symbiotic fermentation of Kombucha. The phylogenetic tree is shown in Figure 4.

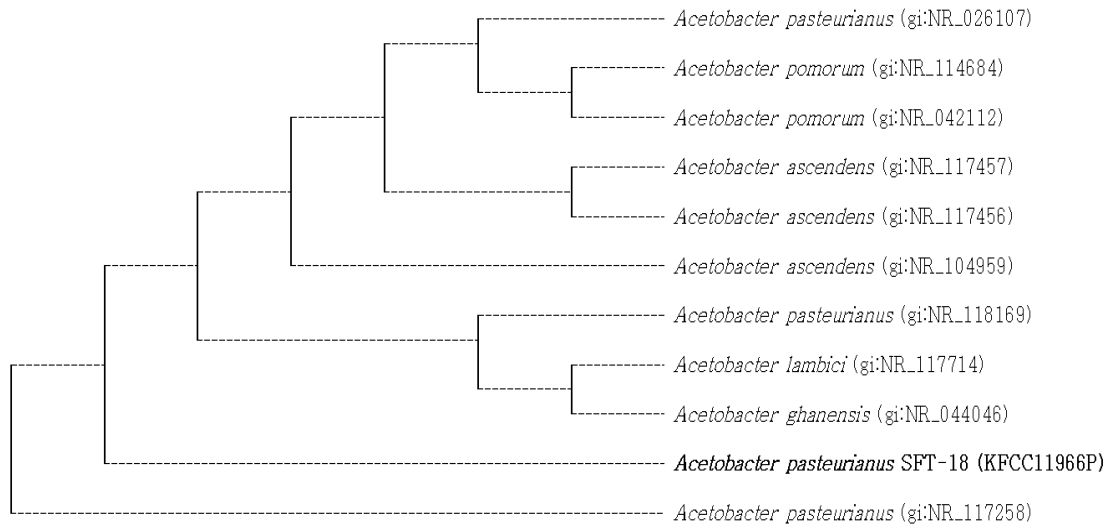

Figure 4. Phylogenetic tree based on 16S rRNA region gene sequences between *Acetobacter pasteurianus* SFT-18 strain and related species.

3.3.2. Content of Gluconate and Glucuronic Acid

The production of functional substances by *Gluconobacter* sp. results from chemical and biological oxidation of glucose into gluconic acid, glucuronic acid, and others. Elimination of many types of toxic substances by glucuronic acid, including exogenous chemicals and excessive steroid hormones, from the human body via the urinary system has been reported [47]. In addition, it can be converted into glucosamine, which is beneficial in the treatment of osteoarthritis and is also known as a precursor of vitamin C biosynthesis [48]. The usefulness of sodium gluconate obtained by the conversion of gluconic acid via microbial fermentation for application in various industries including food and beverage, pharmaceuticals, and others has been reported [49,50].

A culture solution was used at a fermentation temperature of 30 °C based on fermentation characteristics (pH, titratable acidity, and viable cell count) to determine the content of gluconate and glucuronic acid in the isolated sample of *Gluconobacter* sp. The content of gluconate was 25.31 and 25.49 mg/mL, and the content of glucuronic acid was 10.15 and 10.32 mg/mL in *Glu. oxydans* SFT-26 and 27, respectively. Jayabalan et al. [51] reported a glucuronic acid content of 2.33 g/L, and the highest content was detected in Kombucha made from black tea on the 12th day of fermentation. Chen and Liu [52] reported that a glucuronic acid content of approximately 10.0 g/L was detected between the 10th and 20th days and 39.0 g/L was detected on the 60th day after fermentation of Kombucha. A faster rate of glucuronic acid production, as well as a higher overall production amount, was obtained for the two strains (*Glu. oxydans* SFT-26, 27) identified in this study, compared to reports in the existing literature [51,52], both in terms of fermentation time and production rate. However, further verification is required to determine more clearly the impact of Kombucha composition and complex fermentation on changes in content. Therefore, *Glu. oxydans* SFT-27 (Accession; NR_026118.1, Description; *Glu. oxydans*, Length; 1476, Start; 18, End; 1465, Coverage; 98, Bit; 2663, E-Value; 0.0, Match/Total; 1447/1449, Pct. (%); 99.9) can be considered suitable in the production of Kombucha and for enhancing the functionality due to its exceptional capacity for acid resistance and metabolite production. The phylogenetic tree is shown in Figure 5.

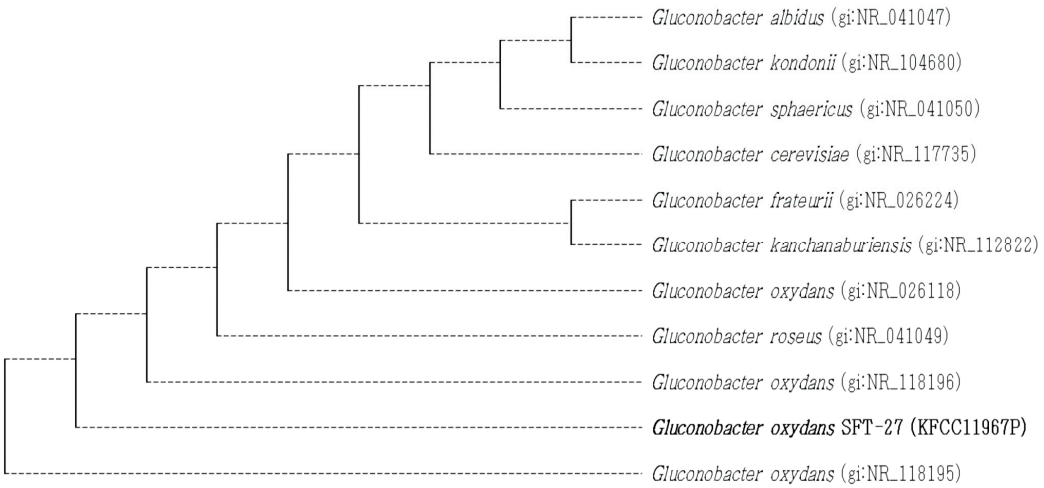

Figure 5. Phylogenetic tree based on 16S rRNA region gene sequences *between Gluconobacter oxydans* SFT-27 strain and related species.

The dominant bacteria in the Kombucha culture belong to the genera *Acetobacter* and *Gluconobacter*, known for producing acetic acid and gluconic acid, respectively [13]. In this study, microbial strains suitable for the complex microbial culture used in the production of Kombucha (SCOBY), including *A. pasteurianus* SFT-18 and *Glu. oxydans* SFT-27, both confirmed for their capacity for producing acetic and gluconic acid, were selected. *A. xylinum*, *A. pasteurianus*, *A. acetic*, *B. gluconicum*, and *Glu. oxydans* were predominantly detected in the currently analyzed Kombucha cultures, and other studies reported similar results [13]. Based on these findings, the two selected strains were frequently detected in Kombucha, indicating their potential for use in the production of Kombucha.

A. pasteurianus, with its high potential in the production of acetic acid [53] and its capacity for producing bacterial cellulose (due to the results of this study), may provide an optimal environment for producing Kombucha [54]. In addition, *Glu. oxydans* [55] may have an important function in conveying various functional and bioactive effects [56] due to the presence of products such as gluconic acid and glucuronic acid (supporting the results of this study).

The main organic acids found in Kombucha include gluconic acid and acetic acid [57], which are known as major compounds contributing to development of the flavor and

quality of Kombucha [58]. Gluconic acid is associated with the drink's pleasant sour taste, while acetic acid is responsible for an astringent and acidic off-flavor. Wang et al. [59] reported the potential utilization of *A. pasteurianus* and the enhanced sensory properties of major organic acids in mixed cultures (*Acetobacter* and *Gluconobacter strain*). In addition, mass production of nutritious Kombucha with consistent quality also poses challenges. Obvious differences in the production of major organic acids such as acetic acid and gluconic acid can be observed depending on the type of acetic acid bacteria, and, depending on the complex microbial culture conditions, it can be a major variable affecting the quality of the Kombucha. Therefore, to ensure the manufacture of commercially usable Kombucha, as well as reproducibility, which is the final objective of this study, we plan on building infrastructure for complex microbial culture of the two selected types of acetic acid bacteria, lactic acid bacteria, and yeast.

4. Conclusions

The mass production of nutritious Kombucha with consistent quality presents several challenges. In addition, the type of AAB is known to influence organic acids such as gluconic acid and acetic acid, which are critical factors in determining the quality of Kombucha during the culturing of the microbial complex. Therefore, in this study, two strains determined to be suitable in the production of Kombucha were selected from among the isolated acetic acid bacteria. The results showed that an optimum temperature range of 30–35 °C was suitable in the fermentation of acetic acid bacteria. Regarding the change in pH and titratable acidity, the lowest pH and highest titratable acidity were detected in *Acetobacter pasteurianus* SFT-18 (pH 3.85, 1.68%) and *Gluconobacter oxydans* SFT-27 (pH 3.56, 0.79%). Regarding the change in viable cell count according to fermentation temperature, a high viable cell count was detected at different incubation temperatures in the *Acetobacter pasteurianus* SFT-18 strain. The *Gluconobacter oxydans* SFT-27 strain contained 10.32 mg/mL of glucuronic acid and 25.49 mg/mL of gluconate. This study was conducted to select acetic acid bacteria for use in the production of Kombucha. However, because the production of Kombucha is achieved through a complex fermentation process involving various microorganisms, it must contain all three types of microorganisms: yeast, lactic acid bacteria, and acetic acid bacteria. Thus, a selection study based on the specific quality characteristics of each microorganism, including yeast and lactic acid bacteria, was conducted. The results demonstrated that *Saccharomyces cerevisiae* SFT-71 (microorganism deposit number: KFCC11969P, Korean Culture Center of Microorganisms) and *Leuconostoc mesenteroides* SFT-45 (microorganism deposit number: KFCC11968P, Korean Culture Center of Microorganisms) were the yeast and lactic acid bacteria, respectively, most suitable for use in complex fermentation. Future research on the manufacturing of Kombucha, including studies on the composition of a complex microbial culture matrix in carefully selected strains, is anticipated.

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