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Effect of Glasswort (*Salicornia herbacea* L.) on Microbial Community Variations in the Vinegar-making Process and Vinegar Characteristics

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Three types of *nuruk* were made from rice, wheat, and a rice–glasswort (6:4) mixture. *Nuruk*, *makgeolli*, and vinegar were manufactured with rice *nuruk* (RN), wheat *nuruk* (WN), and rice–glasswort *nuruk* (RGN). The variable region of 18S or 16S rDNA amplified with genomic DNA extracted directly from *nuruk*-, *makgeolli*-, and vinegar-making cultures was analyzed *via* temperature gradient gel electrophoresis (TGGE). The sequence of the 18S rDNA variable region extracted from the TGGE gel for *nuruk* was 99% homologous with *Aspergillus* sp. and that for the *makgeolli*-making culture was 99% homologous with *Saccharomyces* sp. and *Saccharomycodes* sp. The sequence of the 16S rDNA variable region extracted from TGGE gel for the vinegar-making culture was 98% homologous, primarily with the *Acetobacter* sp. The eukaryotic and prokaryotic diversities in the *nuruk*-, *makgeolli*-, and vinegar-making cultures was not significantly altered by the addition of glasswort. Prokaryotic diversity was higher than eukaryotic diversity in the *nuruk*, but eukaryotic diversity was higher than prokaryotic diversity in the *makgeolli*-making culture, on the basis of the TGGE patterns. No 18S rDNA was amplified from the DNA extracted from the vinegar-making culture. The diversity of the microbial community in the process from *nuruk* to vinegar was slightly affected by the type of raw material utilized for *nuruk*-making. The saccharifying activity and ethanol productivity of *nuruk*, polyphenol content in *makgeolli*, and acetic acid and polyphenol content in the vinegar were increased as a result of the addition of glasswort. In conclusion, the glasswort may be not simply an activator for the growth of microorganisms during the fermentation of *nuruk*, *makgeolli*, or vinegar, but also a nutritional supplement that improves the quality of vinegar.

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Traditionally, Korean *nuruk* is made from wheat by natural inoculation with airborne microorganisms consisting mostly of fungi, yeasts, and various bacteria, in which raw materials were not pasteurized or sterilized [11, 28, 33]. Crushed or ground wheat and barley have also been utilized as raw materials for *nuruk*-making [12]. During the process of brewing Japanese *sake*, rice starch is biochemically converted to glucose by the saccharifying enzymes produced by *koji* (*Aspergillus oryzae*), and the glucose is biologically converted to ethanol *via* the fermentation metabolism of *sake* yeast (*Saccharomyces cerevisiae*) [30, 32]. The *koji* may function similarly to *nuruk*, based on the saccharification of rice starch in the *makgeolli*- or *sake*-making process. The nutrient ingredients of rice are quite similar to those of wheat, according to the Food Composition Table (7th Ed) presented by the Korean Rural Resources Development Institute [26]; however, the minerals, vitamins, and protein contents of rice are 20~40% lower than those of wheat. Glasswort contains relatively high minerals, like seaweed, but substantially lower protein and carbohydrate than are contained in rice and wheat [21]. Glasswort has been reported to harbor a variety of physiologically active compounds: namely, betaine, taurine, polyphenol, and antioxidants [7 8, 13, 17]. The physiologically active compounds and minerals may activate microorganisms for *nuruk*-making, *via* which the biochemical and biological functions of *nuruk* for the fermentation of *makgeolli* may be improved.

Glasswort extraction or powder has been utilized for a variety of food- or cosmetic-making processes *via* mixing or addition [2, 9, 16]. The characteristics of *nuruk* and *makgeolli* may be determined in accordance with the physiological activity of microorganisms grown in the *nuruk*- or *makgeolli*-making cultures. The nutrients of glasswort added to *nuruk*-making cultures can be extracted effectively by

microorganisms, and the growth of microorganisms may be activated by this process. The long-term contact of glasswort with microorganisms in the process from *nuruk* to vinegar may prove helpful for the extraction of insoluble or delayed-soluble compounds.

The diversity or variation of the microbial communities grown in *nuruk*-, *makgeolli*-, and vinegar-making cultures is difficult to estimate *via* the isolation of microorganisms. For the most probable detection of the microorganisms from the *nuruk*-, *makgeolli*-, and vinegar-making cultures, the TGGE technique was employed. DNA extracted directly from the *nuruk*-, *makgeolli*-, and vinegar-making cultures was amplified using the specific primers for the 16S (18S) rDNA and variable region of 16S (18S) rDNA.

In this study, the characteristics of *nuruk*, *makgeolli*, and vinegar made using rice nuruk (RN), wheat *nuruk* (WN), and rice-glasswort *nuruk* (RGN) were compared on the basis of specific indices in order to estimate the effects of glasswort on the characteristics of vinegar. The diversity or variation in the eukaryotic and prokaryotic communities grown in the *nuruk*-, *makgeolli*-, and vinegar-making cultures were analyzed on the basis of the sequence homologies of DNAs extracted from the TGGE gels.

Materials and Methods

Chemicals

All chemicals were purchased from the Korean branch of Sigma-Aldrich (St. Louis, MO, U.S.A.) with the exception of the ingredients utilized to prepare the medium.

Desalting and Grinding of Glasswort

Dried glasswort purchased from Buan Hamcho (Buan, Cheonranam-do, Korea) was soaked for 30 min in running tap water for washing and desalting. The desalted glasswort was then dried under sunlight for 24 h, and ground to a particle size of less than 50 mesh using a ceramic ball mill (SW-BM117, 11.5-l volume; SW Eng, Seoul, Korea).

Preparation of *Nuruk*

Roughly ground (less than 50 meshes) wheat and rice were moistened with distilled water and cast in round-shaped molds (diameter 25 cm ×height 5 cm). The ratio of ground rice and glasswort was adjusted to 6:4 based on weight. The moistened mixture of rice and glasswort (rice–glasswort mixture) was then cast in the same manner as were the others. The prepared *nuruk*-making materials were then incubated for 80 h at 30°C under unventilated conditions, and then incubated for 7 to 10 days at 25°C under ventilated conditions until they had naturally and completely dried.

Saccharifying Activity of *Nuruk*

Approximately 100 g of ground *nuruk* was suspended in tap water to prepare 1,000 ml of slurry. The slurry was then shaken for 60 min at 4°C and 200 strokes to extract the soluble proteins, and then centrifuged for 30 min at 4°C and 5,000 ×*g*. Then 1 g of soluble starch was added to 100 ml of the *nuruk* supernatant and incubated for 30 min at 30°C with shaking (120 rpm). The protein concentration was determined with Bradford reagent (BioRad, Hercules, CA, U.S.A.) and bovine serum albumin (BioRad, Hercules, CA, U.S.A.). Glucose generated by the saccharifying enzyme of *nuruk* extract was determined *via* HPLC.

Preparation for Polyphenol Content Analysis

The *makgeolli*-making culture, which was incubated for 7 days, was centrifuged for 60 min at 4°C and 5,000 ×*g* to obtain clear supernatants. The supernatants were then filtered with a membrane filter (pore size 0.22 μm) and diluted with double-distilled water in the range of a standard curve. The vinegar-making culture, which was incubated for 50 days, was centrifuged for 60 min at 4°C and 5,000 ×*g* to obtain the clear supernatants.

Polyphenol Content Determination

Polyphenol contents were determined *via* the Prussian Blue spectrophotometric method [9, 22]. First, 3.0 ml of 0.1 M $FeCl_3$ in 0.1 M HCl was added to 1 ml of the supernatant, followed immediately by the timed addition of 3.0 ml of freshly prepared 0.008 M $K_3Fe(CN)_6$. The absorbance was then measured *via* spectrophotometry (Shimadzu UV-1601, Tokyo, Japan) at 720 nm for 10 min after the introduction of the reagents. A standard curve was prepared to express the results as tannic acid equivalents; that is, the quantity of tannic acid (mg/l) required to achieve a color intensity equivalent to that of the polyphenols after blank correction.

Brewing of *Makgeolli*

Approximately 2 kg of rice soaked in water for 48 h was steamed for 60 min and cooled to room temperature. Then 500-g measures of RN, WN, and RGN powder were separately added to the steamed rice samples and mixed thoroughly. The mixtures of *nuruk* and steamed rice were then placed into glass carboys (Pyrex No 1596, 19L; Corning, U.S.A.) and incubated for 7 days at 25°C, after the addition of 3.0 l of boiled tap water to each. HPLC analysis was subsequently conducted in order to evaluate ethanol production from the *makgeolli* cultures. The final products were designated RN-, WN-, and RGN-*makgeolli*, based on the materials used for *nuruk*-making.

Brewing of Vinegar

Makgeolli that was prepared with RGN, WN, and RN was filtered with 50-mesh filters, after which the filtrate was diluted 2× with autoclaved distilled water and placed into glass carboys (Pyrex No 1596, 13L; Corning, U.S.A.), and then incubated for 50 days without inoculation of specific vinegar starter. Thereafter, 10 ml of vinegar culture was sampled from the carboys for DNA analysis every 10 days. The final products were designated as RGN-, WN-, and RN-vinegar based on the *makgeolli* utilized for vinegar fermentation.

Analysis of Sugar and Alcohol

The ethanol generated from the glucose-defined medium with and without glasswort or *makgeolli* cultures was analyzed *via* HPLC (Young-Lin, Seoul, Korea) using an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, CA, U.S.A.) and a refractive index detector (Young-Lin, Seoul, Korea). The column and detector were adjusted to a temperature of 35°C. The mobile phase was sulfuric acid (0.008 N) and the flow rate was 0.6 ml/min. The samples prepared *via* 30 min of centrifugation at 12,000 ×*g* and 4°C were filtered

through a 0.22-μm-pore membrane filter. Then 20 μl of filtrate was injected into the HPLC injector. The glucose and ethanol concentrations were calculated on the basis of the peak area in the chromatograms generated using standard materials.

Analysis of Organic Acids and Minerals

The organic acid contents of the samples were analyzed *via* HPLC (Beckman, Coulter System Gold, Brea, CA, U.S.A.) with an ion-exclusion column (Shodex, Rspak KC-811; Showa Denko, Tokyo, Japan) and a refractive index detector (Shodex, RI-101; Showa Denko, Tokyo, Japan). The column and detector were adjusted to a temperature of 40°C. The mobile phase was $HClO_4$ (6 mM) with the flow rate adjusted to 0.8 ml/min. The samples prepared *via* 30 min of centrifugation at 12,000 ×*g* and 4°C were filtered through a 0.22-μm-pore PVDF membrane filter. Then 30 μl of filtrate was then injected into the HPLC injector. The organic acid concentrations were calculated on the basis of the peak area in the chromatograms generated using standard materials.

The minerals were analyzed using an Inductively Coupled Plasma (ICP) spectrophotometer (Spectro CCD-ICP; Boschstr, Kleve, Germany). The supernatant obtained from the *makgeolli* was filtered with a 0.22-μm-pore PVDF membrane filter and diluted 20-fold. The diluted filtrate was then directly injected into the ICP injector under specific wavelengths for Mg (279.553 nm), Na (589.592 nm), K (766.491 nm), Ca (396.847 nm), and Fe (2579.941 nm). The concentrations of minerals were calculated on the basis of the absorbance obtained with standard materials (AccuTrace Reference Standard; AccuStandard, New Haven, CT, U.S.A.) and at standard dilution rates.

Temperature Gradient Gel Electrophoresis

The 16S rDNA amplified from chromosomal DNA was employed as a template for the preparation of the TGGE sample (16S rDNA variable region). A variable region of 16S rDNA was amplified with forward primer (eubacteria, V3 region) 341f 5'-CCTACGGGAGGC AGCAG-3' and reverse primer (universal, V3 region) 518r 5'-ATTACCGCGGCTGCTGG-3'. A GC clamp (5'-CGCCCGCCGCG CGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') was attached to the 5'-end of the GC341f primer [18]. The procedures for PCR and DNA sequencing were identical to the 16S rDNA amplification conditions, with the exception of the annealing temperature.

The 18S rDNA amplified from chromosomal DNA was utilized as a template for the preparation of the TGGE sample (18S rDNA variable region). A variable region of 18S rDNA was amplified with forward primer (wide range of fungal taxa) EF3 5'-TCCTCTAAAT GACCAAGTTTG-3' and reverse primer (wide range of fungal taxa) EF4 5'-GGAAGGGRTGTATTTATTAG-3'. A GC clamp (5'-CGCCC GCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGG-3') was attached to the 5' end of NS-3 (5'-GCAAGTCTGGTGCCAGC AGCC-3') [22, 29]. The procedures for PCR and DNA sequencing were identical to the 18S rDNA amplification conditions, with the exception of the annealing temperature. The TGGE system (Bio-Rad, Dcode, Universal Mutation Detection System, U.S.A.) was operated in accordance with the manufacturer's specifications. Aliquots (45 ml) of the PCR products were electrophoresed in gels containing 8% acrylamide, 8 M urea, and 20% formamide with a 1.5× TAE buffer system at a constant voltage of 100 V for 12.5 h and then at 40 V for 0.5 h, applying a temperature gradient of 39 to 52°C. Prior to electrophoresis, the gel was equilibrated to the temperature gradient for 30 to 45 min.

Amplification and Identification of TGGE Bands

DNA was extracted from the TGGE band and purified using a DNA gel purification kit (Accuprep, Bioneer, Korea). The purified DNA was then amplified with the same primers and procedures employed for TGGE sample preparation, in which the GC clamp was not attached to the forward primer. The species-specific identities of the amplified variable 16S rDNA and 18S rDNA were determined on the basis of sequence homology, according to the information in the GenBank database system.

RESULTS

Functional Character of *Nuruk*, *Makgeolli*, and Vinegar

Makgeolli fermented with RN, WN, and RGN was utilized as a source for vinegar fermentation. The effects of glasswort on the saccharifying activity of the completed *nuruk*, alcohol and polyphenol contents in the completely fermented *makgeolli*, and acetic acid and polyphenol contents in the completely fermented vinegar were estimated, as shown in Table 1. The ethanol content in *makgeolli* may be proportional to the saccharifying activity of *nuruk*, and the acetic acid content in vinegar may be proportional to the ethanol concentration. Accordingly, the biochemical function of *nuruk* may be a crucial factor in the determination of vinegar characteristics. Glasswort may be a factor in activating the growth of the microorganisms capable of saccharifying

Table 1. Effects of glasswort on the functional character of products manufactured in each step from the *nuruk*- to vinegar-making process.

Nuruks	Quantitative estimation of products in each step				
	[a]Saccharifying activity of *nuruk* (g/l)	[b]Ethanol in *makgeolli* (g/l)	[c]Polyphenol in *makgeolli* (mg/l)	[d]Acetic acid in vinegar (g/l)	Polyphenol in vinegar (mg/l)
RN	1.41±0.1	93.3±2.4	21.4±2.1	52.6±3.6	9.3±0.8
WN	1.39±0.1	91.7±3.5	21.9±1.8	53.7±2.8	8.9±0.4
RGN	2.02±0.1	123.3±3.2	68.4±3.3	70.8±3.2	56.7±3.8

[a]Glucose produced from starch by 10% (w/v) of *nuruk* slurry for 30 min.
[b]Ethanol produced from rice by three different *nuruks* for 7 days.
[c]Polyphenol content in supernatant of *makgeolli* obtained by centrifugation.
[d]Acetic acid produced from *makgeolli* by fermentation for 50 days.

starch and a source for additional polyphenols in the *makgeolli* and vinegar. If the glasswort was added to the *makgeolli* or vinegar, the nutrients in the glasswort may be extracted simply with ethanol or acetic acid, but may not be an important factor in the activation of the microorganisms. Glasswort may induce the microorganisms to generate more saccharifying enzymes in the *nuruk*-making cultures, based on the differences in glucose production activity between RGN and RN. The differences in ethanol production activities between RGN and RN may be caused by differences in sugar concentration and glasswort's effects on yeast activity. Experimentally, *Saccharomyces cerevisiae* generated 9.7 g/l of ethanol, but 72.4 g/l of ethanol *via* the addition of 20 g/l of glasswort in 180 g/l of glucose solution for 48 h. The *makgeolli* fermented by RGN may contain glasswort extract and higher ethanol contents than in the other *makgeolli* variants fermented with RN or WN. This causes the concentration of acetic acid produced from RGN-*makgeolli* to be higher than that from the RN- or WN-*makgeolli*.

Table 2. Concentration of organic acids and minerals contained in vinegar that were produced by additional fermentation of RN-, WN-, and RGN-*makgeolli*.

Contents (mg/l)	Vinegars		
	RN	WN	RGN
Citric acid	-	-	1,614±58
Malic acid	-	-	1,793±96
Succinic acid	-	-	735±42
Lactic acid	4,092±120	4,314±156	2,554±89
Acetic acid	52,619±288	53,753±302	70,169±532
Pyroglutamic acid	401±16	292±13	2,214±109
Ca	26.85±1.5	22.21±1.3	77.78±2.4
Fe	0.81±0.1	1.45±0.2	10.25±0.8
K	172.9±6.3	170.7±4.1	329.5±8.1
Mg	74.4±1.5	84.2±1.6	207.5±1.8

All data are mean values obtained by three identical experiments and analyses.

Microbial Community Diversity in the *Nuruk*-Making Cultures

The eukaryotic and prokaryotic microorganisms grown in the *nuruk*-making cultures were analyzed *via* TGGE, as shown in Fig. 1 and 2. Eukaryotic diversity was not especially affected by the addition of glasswort (Fig. 1). The predominant eukaryotes in the *nuruk*-making cultures were identified as *Aspergillus oryzae*, the DNA of which was extracted from the major TGGE bands. The prokaryotic diversity was increased slightly by the addition of glasswort (lane 1, Fig. 2). The DNA sequence extracted from the TGGE gel was more than 98% homologous with *Pediococcus* sp. (Gram-positive lactic acid bacterium), *Kyotococcus* sp. (soil bacterium), *Micrococcus* sp. (soil bacterium), *Bacillus* sp. (soil bacterium), and *Streptomyces* sp. (soil bacterium), respectively, as shown in Table 3. *Pediococcus acidilactici* (band no. 4) was observed commonly in RGN and RN, *Micrococcus* sp. (band no. 7) and *Streptomyces* sp. (band

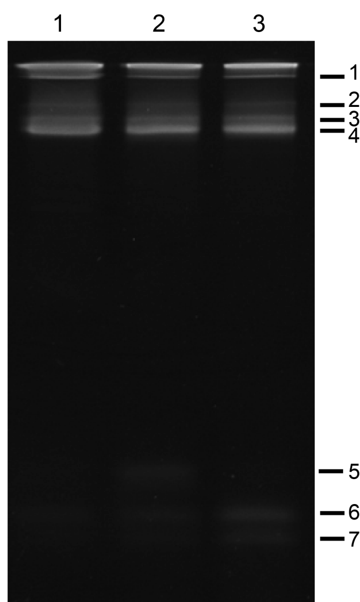

Fig. 1. TGGE pattern of the 18S rDNA variable region amplified with genomic DNA extracted from RGN (lane 1), WN (lane 2), and RN (lane 3).
The homologous microorganisms with DNAs extracted from the numbered bands are arranged in the order of the band numbers in Table 3.

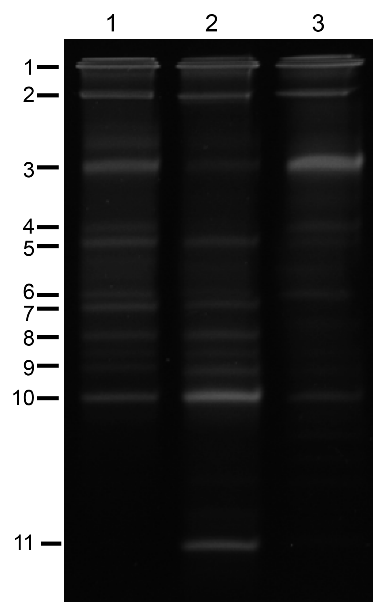

Fig. 2. TGGE pattern of the 16S rDNA variable region amplified with genomic DNA extracted from RGN (lane 1), WN (lane 2), and RN (lane 3).
The homologous microorganisms with DNAs extracted from the numbered bands are arranged in the order of the band numbers in Table 3.

Table 3. Microorganisms that are homologous with DNA extracted from the numbered bands in the TGGE performed for analysis of microbial community variation during cultivation of *nuruk* (Fig. 1 and 2).

Band No.	Eukaryotes		Prokaryotes	
	Genus or species (Accession No.)	Homology (%)	Genus or species (Accession No.)	Homology (%)
1	*Aspergillus oryzae* (AP007155)	99	Uncultured bacterium (EU471728)	99
2	*Aspergillus oryzae* (AB226217)	99	Uncultured bacterium (EU776571)	99
3	*Aspergillus niger* (AJ878650)	98	Uncultured bacterium (EU472921)	98
4	*Aspergillus oryzae* (AB226239)	100	*Pediococcus acidilactici* (AB018212)	100
5	-	-	*Kyotococcus* sp. (DQ107403)	99
6	-	-	*Pediococcus acidilactici* (FJ751795)	99
7	-	-	*Micrococcus* sp. (EU358178)	100
8	-	-	*Streptomyces* sp. (EU554280)	100
9	-	-	*Bacillus subtilis* (DQ195067)	98
10	-	-	*Bacillus licheniformis* (GQ169102)	99
11	-	-	*Streptomyces* sp. (EU554280)	100

no. 8) were observed commonly in RGN and WN, and *Streptomyces* sp. (band no. 11) was observed in only WN. From these results, we conclude that some of the bacterial species grown in the *nuruk*-making cultures originated from a specific raw material (rice, wheat, or glasswort).

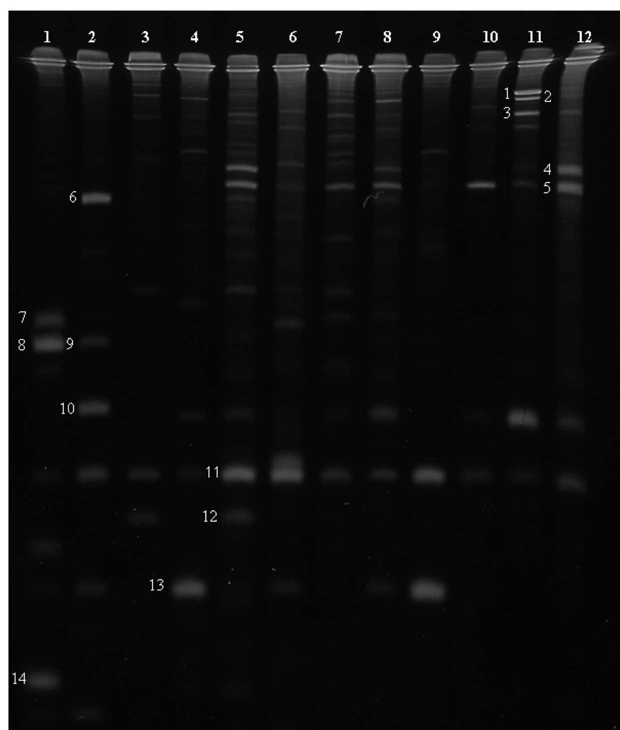

Fig. 3. Time-coursed variation of the TGGE pattern of the 18S rDNA variable region amplified with genomic DNA extracted from the *makgeolli* fermented by RGN (lanea 1,4,7,10), WN (lanes 2,5,8,11), and RN (lanes 3,6,9,12) at the 1st day (lanes 1,2,3), 3rd day (lanes 4,5,6), 5th day (lanes 7,8,9), and 7th day (lanes 10,11,12) of incubation time.
The homologous microorganisms with DNAs extracted from the numbered bands are arranged in the order of the band numbers in Table 4.

Microbial Community Diversity in *Makgeolli*-Making Cultures

The *makgeolli*-making culture condition must be anaerobic and saturated with carbon dioxide during the active growth phase of yeast, from the 2nd to 5th days of incubation time, thus inhibiting or limiting prokaryotic growth. As is shown in Fig. 3 and 4, the prokaryotic diversity was substantially

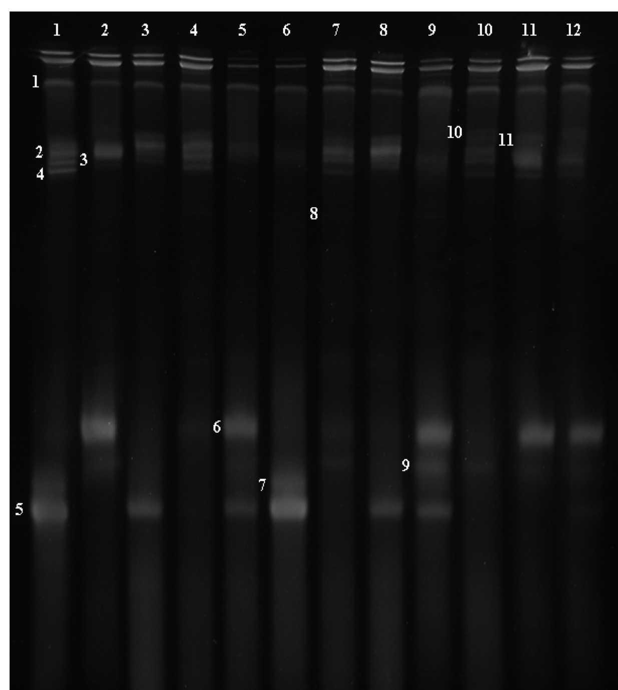

Fig. 4. Time-coursed variation of the TGGE patters of the 16S rDNA variable region amplified with genomic DNA extracted from rice-*makgeolli* fermented by RGN (lanes 1,4,7,10), WN (lanes 2,5,8,11), and RN (lanes 3,6,9,12) at the 1st day (lanes 1,2,3), 3rd day (lanes 4,5,6) 5th day (lanes 7,8,9), and 7th day (lanes 10,11,12) of incubation time.
The homologous microorganisms with DNAs extracted from the numbered bands are arranged in the order of the band numbers in Table 4.

Table 4. Microorganisms that are homologous with DNA extracted from the numbered bands in the TGGE performed for analysis of microbial community variation during cultivation of *makgeolli* (Fig. 3 and 4).

Band No.	Eukaryotes		Prokaryotes	
	Genus or species (Accession No.)	Homology (%)	Genus or species (Accession No.)	Homology (%)
1	Unidentified		Uncultured *Bacillus* sp. (GQ180959)	98
2	Uncultured eukaryote (GU297613)	99	Uncultured bacterium (AM273913)	99
3	Unidentified		Uncultured bacterium (FJ674120)	99
4	Uncultured eukaryote (EU860642)	99	Uncultured bacterium (GQ866181)	99
5	Uncultured eukaryote (AB505568)	99	Uncultured bacterium (AM292547)	99
6	Unidentified		Uncultured *Bacillus* sp. (GQ471880)	99
7	Unidentified		Unidentified	
8	Unidentified		Unidentified	
9	*Candida* sp. (EF550423)	98	Uncultured bacterium (EF521194)	99
10	*Saccharomycodes ludwigii* (AY046261)	99	Unidentified	
11	*Saccharomyces* sp. (GU213443)	100	Unidentified	
12	Uncultured eukaryote (FN394745)	99	-	-
13	Uncultured fungal clone (FJ779910)	99	-	-
14	Unidentified		-	-

lower than the eukaryotic diversity, as anticipated. In particular, the eukaryotic diversity was maximally increased at the 3rd and 5th days of incubation. The majority of 18S rDNA extracted from the TGGE gels was identified with the uncultured eukaryotes. The DNA that was observed commonly (lane 11) and mostly (lane 10) in the TGGE gels for all samples obtained from the *makgeolli*-making cultures during 7 days of incubation was identified as that of *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii*, respectively. Some TGGE bands (nos. 6, 7, 8, and 14) were observed in the *makgeolli* culture at the initial incubation time and then disappeared, but some of them (nos. 10 and 11) were maintained during incubation for 7 days of incubation however, some of them (nos. 1, 2, 3, 4, and 5) appeared late at the mid or end stage of incubation. These results present a clue that some of uncultured eukaryotes grown in the *makgeolli* culture may be dependent upon the environmental condition generated by yeast cells. Meanwhile, the sequences of the DNA bands extracted from TGGE gels for the 16S rDNA variable region were identified primarily as being from uncultured bacteria, with the exception of one that was identified as *Bacillus* sp., and some other unidentified bacteria, as shown in Table 4.

Microbial Community Diversity in Vinegar-Making Culture

DNA was directly extracted from the samples obtained from vinegar-making cultures and then amplified with primers for the amplification of 16S rDNA and 18S rDNA; however, 18S rDNA was not amplified or was weakly detected on agarose gels (data not shown). The amplified 16S rDNA variable region was analyzed *via* TGGE, as shown in Fig. 5. The initial community diversity in the RGN- and WN-vinegar-making cultures was higher than that observed in the RN-vinegar-making culture. However, the majority of DNA extracted from the TGGE gel was identified with the uncultured bacteria, with the exception of *Lactobacillus* sp. (band number 8), *Lactobacillus nagelii* (band number 9), and *Deinococcus* sp. (band number 28), as shown in Table 5. The bacterial community diversities were significantly simplified after 30 days of incubation. *Acetobacter* sp. was spontaneously enriched after 30 days of incubation, but bacterial species other than *Lactobacillus* sp. (band number 8 in Fig. 5A) selectively disappeared.

Organic Acids and Minerals Contained in Vinegars

Organic acids contained in the vinegar may be generated by a variety of organic acid fermentation bacteria, with the exception of acetic acid, which is generated principally by *Acetobacter* sp. or *Gluconobacter* sp. As is shown in Table 2, citric acid, malic acid, and succinic acid were contained selectively in the RGN-vinegar. The acetic acid content of RNG-vinegar was approximately 1.3 times that measured in the RN- and WN-vinegars; however, the lactic acid content was approximately 0.6 times lower. The pyroglutamic acid content of RGN-vinegar was significantly higher than those of the RN- and WN-vinegars. The difference in organic acid contents between the RGN- and RN-vinegars may originate from differences in the microbial growth conditions that may be caused by the addition of glasswort to the *nuruk*-making cultures. The Ca, Fe, K, and Mg contents of RGN-vinegar were higher than those of the RN- and WN-vinegars. In particular, the Fe contents of the RGN-vinegar were approximately 13 and 7 times those of the RN- and WN-vinegars, respectively. The differences in mineral content between the RGN- and RN-vinegars may be caused by the effects of the addition of glasswort to rice during the *nuruk*-making process.

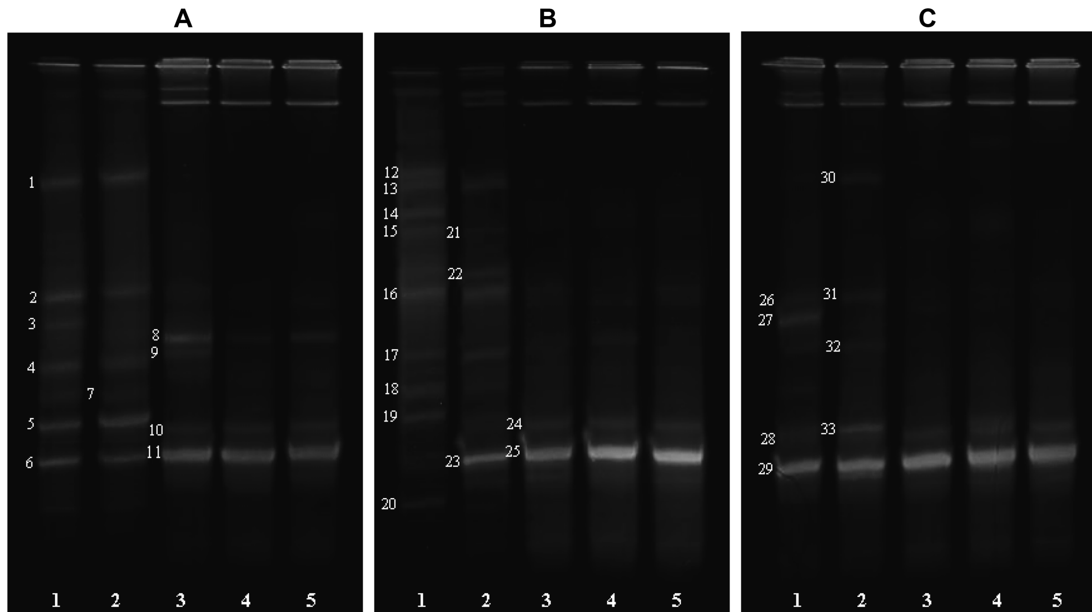

Fig. 5. Time-coursed variation of the TGGE pattern of the 16S rDNA variable region amplified with genomic DNA extracted from RGN- (**A**), WN- (**B**), and RN-vinegar-making culture (**C**) at the 10th day (lane 1), 20th day (lane 2), 30th day (lane 3), 40th day (lane 4), and 50th day (lane 5) of incubation time during 50 days of fermentation.
The homologous microorganisms with DNAs extracted from the numbered bands are arranged in the order of the band numbers in Table 5.

DISCUSSION

For the brewing of vinegar with grains, *nuruk* has to be employed for the biochemical saccharification of starch to glucose and the biological fermentation of glucose to ethanol [6]. The microorganisms grown in *nuruk*-making cultures are difficult to measure quantitatively, but can be indirectly measured based on the saccharifying activity of starch or the fermentation efficiency of ethanol, which may increase in proportion to the growth of microorganisms operating in the *nuruk*-making process [23]. Wheat harbors higher protein, and more diverse and higher concentrations of minerals and vitamins than rice [26]. Nevertheless, the difference in the nutritional contents of wheat and rice was not a cause of the activation or limitation of the functional effects of *nuruk* for the saccharification of starch and the

Table 5. Microorganisms that are homologous with DNA extracted from the numbered bands in the TGGE performed for analysis of microbial community variation during cultivation of vinegar (Fig. 5).

Band No.	Genus or species (Accession No.)	Homology (%)	Band No.	Genus or species (Accession No.)	Homology (%)
1	Uncultured bacterium clone (GQ258076)	99	18	Uncultured bacterium (GQ866160)	99
2	Uncultured bacterium partial 16S rDNA (AM921513)	99	19	Uncultured bacterium (FJ499356)	99
3	*Deinococcus* sp. (EU718060)	99	20	Uncultured *Anaerofilum* (FJ823903)	99
4	Uncultured bacterium (AY939020)	99	21	Uncultured bacterium (AM292550)	99
5	*Deinococcus* sp. (EU710548)	100	22	Uncultured bacterium (GQ372918)	99
6	Uncultured A*cetobacter* sp. (GQ332236)	99	23	*Acetobacter pasteurianus* (GU205100)	99
7	Uncultured bacterium (AY938981)	99	24	*Acetobacter pasteurianus* (AB499842)	100
8	*Lactobacillus* sp. (AB016864)	99	25	*Acetobacter pasteurianus* (GU205100)	100
9	*Lactobacillus nagelii* (AB370876)	100	26	Uncultured bacterium (GQ165198)	99
10	*Acetobacter pasteurianus* (GU205100)	100	27	Uncultured *Acetobacter* (GU198916)	99
11	*Gluconobacter oxydans* (GU205101)	100	28	*Deinococcus* sp. (EU710548)	99
12	Uncultured bacterium clone (EU472679)	99	29	*Acetobacter pasteurianus* (AB499840)	99
13	Uncultured bacterium (GQ467303)	99	30	Uncultured bacterium (EU978605)	99
14	Uncultured *Lactobacillus* (DQ334799)	99	31	Uncultured *Acetobacteraceae* (EF663860)	99
15	Uncultured bacterium (GQ453513)	99	32	Uncultured bacterium (GQ402627)	98
16	Uncultured bacterium (GQ242786)	99	33	Uncultured bacterium (AY882673)	99
17	Uncultured bacterium (EU275375)	99		-	-

fermentation of ethanol. Glasswort contains lower organic compound contents (carbohydrate, protein and lipid), but higher minerals and physiologically active compounds (polyphenol, taurine, and betaine) than are contained in rice and wheat [2, 21, 26]. Accordingly, the addition of glasswort to rice may cause the supplementation of minerals and physiologically active compounds. The difference between the biochemical and biological activities of RN and RGN utilized in the vinegar-making processes is a clue that glasswort functions as a physiological activation factor rather than a nutritional supplement for microorganismic growth, considering the differences in nutritional contents between wheat and rice [14, 27].

Some of the nutritious ingredients contained in glasswort may be factors for the activation of microorganismic growth, but not for the differentiation of community or the diversity of microorganisms [25]. It can be surmised that the diversity of microorganisms generating saccharifying enzymes and fermenting *makgeolli* and vinegar may be determined by the environmental conditions of the places where the *nuruk* is produced, rather than the nutritional ingredients of the raw materials. Fungi belonging to the *Aspergillus* genus were commonly identified in RN, WN, and RGN, independently of nutritional contents; however, some of the microorganisms isolated from other *nuruks* were identified as *Rhizopus oryzae*, *Rhizopus koji,* and *Aspergillus coreanus*, which are typical starch-saccharifying fungi grown in the *nuruk* [15, 28, 33]. Some of the microorganisms grown in the *makgeolli*-making cultures may very possibly have been contaminated by the surrounding environment and *makgeolli*-making operators, but some of them may have originated from the *nuruk* [3]. *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii*, the DNA of which was detected in all or most of the *makgeolli*-making cultures regardless of the *nuruk* type, but was not detected commonly in the RN, WN, and RGN, may have initially been contaminated by the environment [24]. The prokaryotes detected in the *makgeolli*-making cultures were also not detected in the *nuruk*, with the exception of *Bacillus* sp. The environmental conditions pertinent to the *makgeolli*-making cultures may not be appropriate for prokaryotic growth, because the ethanol and carbon dioxide, which increased in proportion to the incubation time, may be a factor in the inhibition of the prokaryotes [1, 5, 19, 25]. The vinegar-making cultures cannot be readily contaminated by airborne microorganisms or from the surrounding environments, because both ethanol and acetic acid are difficult to catabolize under anoxic or oxygen-limited conditions [4, 20, 31]. The increase in acetic acid in proportion to incubation time may also be a cause of the limitation of microbial contamination as the result of decreasing pH.

The diversities of microorganisms grown in the *nuruk*-, *makgeolli*-, and vinegar-making cultures cannot be standardized on the basis of the characteristics of the foods that are fermented naturally by surrounding microorganisms [10]. The uncultured eukaryotes observed in the *makgeolli*-making cultures are, like yeast, supposed to perform specific functions on the basis of the increases or reductions in the diversity over the course of the incubation time. The significantly lower diversity of the prokaryotes observed in the *makgeolli*-making cultures as compared with the *nuruk*-making cultures may be caused by the different conditions of the *nuruk* and *makgeolli* for prokaryotes. Most of the prokaryotes growing in the *nuruk* are routinely exposed to air, but those growing in the *makgeolli* must be in contact with at least some air in the initial stages before the activation of ethanol fermentation.

In conclusion, the characteristics of vinegar are dependent on both the nutritional ingredients of raw materials that function as growth factors and the microorganisms that are responsible for the fermentation necessary for the successful preparation of *nuruk*, *makgeolli*, and vinegar cultures. The addition of glasswort to *nuruk*-making cultures may not only be a factor in the activation of microorganism growth, but also a nutritional supplement in the vinegar-making process. The human health-promoting materials employed in foods after fermentation may be considered food additives, but those present before fermentation may activate microorganism growth, improve food characteristics, and increase the levels of physiologically active compounds. In this study, the diversity of eukaryotes and prokaryotes in the *nuruk*-, *makgeolli*-, and vinegar-making cultures was initially analyzed *via* the TGGE technique. By applying the TGGE technique to the natural fermentation of vinegar from *nuruk*, we were able to determine that *Aspergillus* sp., *Saccharomyces* sp., and *Acetobacter* sp. were the principal organisms responsible for *nuruk*-making, *makgeolli* fermentation, and vinegar fermentation, respectively. A variety of uncultured eukaryotes and prokaryotes may generate some metabolites that could influence the flavor and taste of the prepared vinegar. A real-time technique for the monitoring of variations in the eukaryotic and prokaryotic communities to determine the state of fermentation must be developed on the basis of the DNA sequence obtained from the TGGE of microorganisms grown in the *nuruk*-, *makgeolli*-, and vinegar-making cultures. In the future, various types of *nuruk* generated by different makers, and *makgeolli* produced by those *nuruk* variants, will be analyzed *via* the TGGE technique.

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