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| 보 문 |

Microbial Diversity in the Enrichment Cultures from the Fermented Beverage of Plant Extract Using Ribosomal RNA Sequence Analysis

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라이보좀 RNA 염기서열 분석을 이용한 집식배양된 식물추출물발효음료의 미생물 다양성

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A beverage was produced by the fermentation of mixed extracts from the various fruits, vegetables, algae, and medical herbs. The physicochemical properties of the fermented beverage of plant extracts (FBPE) and microbial diversity were analyzed in cultures enriched from FBPE using 16S and 26S rRNA gene sequence analyses. The pH, acidity, °brix, reducing sugar, and alcohol contents of the FBPE were determined to be the 3.48, 1.68%, 70.0, 1,026 g/L, and 3.5%, respectively. The most abundant free sugar and organic acid in the FBPE were glucose (567.83 g/L) and tartaric acid (93.68 mg/L), respectively. *Lactobacillus homohiochii* was the predominant species in all enriched culture samples: 100% of the species in 0B (0% sugar) and 40B (40% sugar) libraries and 95.6% of 20B library (20% sugar). *Lactobacillus fructivorans* was detected in the 20B library. The predominant species in the samples of enrichment cultures collected from FBPE with three different sugar concentrations were: *Candida zeylanoides* (45.2%) in the 0Y library (0% sugar), *Candida lactis-condensi* (35.7%) and *C. zeylanoides* (35.7%) in the 20Y library (20% sugar), and *C. lactis-condensi* (38.1%) in the 40Y library (40% sugar). This result may provide a useful frame of reference for further analyses of microbial population dynamics in FBPE.

***Keywords*:** 16S and 26S rRNA genes, fermented beverage of plant extract, lactic acid bacteria, microbial diversity, yeast

Since the beginning of human civilization there has been an intimate relationship between human beings, and the fermentative activities of microorganisms (Blandino *et al*., 2003). Fermented foods make an important contribution to the human diet in many countries because fermentation is an inexpensive technology that preserves food, improves its nutritional value, and enhances its sensory properties (Gadaga *et al*., 1999). Fermentation may also lead to the detoxification and destruction of undesirable factors present in raw foods such as phytates, tannins, and polyphenolics. Fermented foods are produced worldwide using various manufacturing techniques, raw materials and microorganisms. Fermentation process for food production could be divided into four main catagorieo such as alcoholic, lactic, acetic and alkaline fermentation (Soni and Sandhu, 1990).

In foods, microorganisms are often subjected to abiotic stresses caused by technological and ripening processes as well as by the methods used to recover microbial cells from the food

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matrix. In this regard, detailed studies are needed to optimize the recovery and revival of microorganisms and to monitor the evolution of, and relationship among, bacterial and yeast communities in foods (Giraffa and Neviani, 2001). Pre-enrichment and selective enrichment cultures carried out before plating should be adapted and optimized to allow the recovery of less dominant, stressed, and uncultivable bacterial and yeast populations, which are present at low levels (Giraffa and Neviani, 2001).

In the past fifteen years, our knowledge on microbial diversity in mixed microbial communities has dramatically increased due to the use of molecular approaches. Molecular technique provide outstanding tool for the detection, identification, and characterization of microorganisms involved in various environmental and food ecosystems (Giraffa and Neviani, 2001). The rRNA molecules consist of highly conserved domains interspersed with variable regions. Thus, the comparative analysis of these sequences is a powerful means for inferring phylogenetic relatedness among organisms (Wintzingerode *et al*., 1997; Cho and Seo, 2007; Cho *et al*., 2009). Methods that have arisen from this work include cloning and sequence of rRNA genes. In particular, the rRNA sequences together with chemotaxonomic and genomic analyses is one of the most powerful methods for inferring the relationships between genera or between species belonging to a genus (Cho and Seo, 2007).

The beverage was produced by fermenting a mixture of extracts from approximately 50-60 types of fruits and vegetables. The extract was obtained by osmotic pressure using high concentrates of sucrose (over 55 brix°) and was fermented using microorganisms such as *Leuconostoc* spp., *Zygosaccharomyces* spp., and *Pichia* spp. (Okada *et al*., 2006). In this study, clone libraries were constructed using ribosomal RNA (rRNA) genes amplified from cultures enriched from the fermented beverage of plant extract (FBPE). Six libraries were analyzed and compared to 16S and 26S rRNA gene sequences obtained from enrichment cultures under various growth conditions.

Materials and Methods

Materials, chemicals, and instruments

The fermented beverage of plant extract (FBPE) was obtained from the Farming Cooperation Mario, in Goseung-gun, Korea. Three standard sugars, including, glucose, fructose, and sucrose, were purchased from Sigma-Aldrich Chemical Co. (USA). Nine organic acids, namely oxalic acid, tartaric acid, malic acid, ascorbic acid, lactic acid, acetic acid, citric acid, succinic acid, and glutaric acid were obtained from Sigma-Aldrich Chemical Co.. The analytical grade water and acetonitrile used for High performance liquid chromatography (HPLC) analysis were purchased from Fisher Scientific (USA). Restriction enzymes and DNA modifying enzymes were purchased from Promega (USA). Other chemicals were purchased from Sigma-Aldrich Chemical Co.. All other reagents were of analytical grade.

UV spectra were measured on Spectro 2D spectrophotometer (Spectronic 2D, Thermo Electron Co., USA). HPLC was performed using an Agilent 1200 series instrument (Agilent Co., Australia) equipped with a quaternary HPLC pump, a degasser, and an Agilent 1200 series diode array (DA) and reflex index (RI) detectors. The free sugars were analyzed on a Polyamine II column (4.6 × 150 mm, 5 μm, YMC Corp., Japan). In addition, the organic acids were analyzed on TSK gel ODS-100V column (4.6 × 250 mm, 5 μm, Tosoh Corp., Japan). Polymerase chain reaction (PCR) was performed using a thermal cycler (model MJ Mini^{TM}; Gradient Thermal Cycler, USA).

Bacteria, plasmids, and media

Escherichia coli DH5α (Real Biotech. Corp., USA) and recombinant *E. coli* cells were cultured at 37℃ in Luria-Bertani (LB) medium or LB medium supplemented with 50 μg/ml ampicillin. The ampicillin was purchased from Sigma-Aldrich Chemical Co.. pGEM-T easy vector (Promega) was used for cloning and sequencing. The LB, Lactobacilli MRS (MRS), and Potato Dextrose (PD) media were purchased from Difco (Becton Dickinson Co., USA).

pH and acidity

The FBPE was filtered to collect the fluid portion and the pH was measured with a pH meter (model 3510, Jenway, UK). A 20 mL aliquot of FBPE filtrate was titrated with 0.1 N NaOH to pH 8.2±0.1 to determine the titratable acidity. The titratable acidity was calculated relative to lactic acid.

Brix, reducing sugar, and alcohol

The brix was measured with a saccharometer (N-1α, Atago Co., Japan). The reducing sugar in the FBPE filtrate was measured with a dinitro-salicylate (DNS)-based method (Miller, 1959), modified to quantify glucose equivalents. Six glucose strandard solutions of 100, 250, 500, 750, and 1,000 mg/L were prepared in deionized water. A 100 μl aliquot of each solution was mixed with 900 μl DNS reagent, bowled at 100℃ for 10 min, and then cooled at 4℃. The absorbance of the solution was measured at 570 nm, and a standard curve was obtained by plotting concentration against absorbance. Similarly, a 100 μl sample of the diluted FBPE filtrate was collected and prepared using the same procedures described, and quantified using the linear regression equation of the glucose standard curve. A 100 ml aliquot of FBPE was mixed with 100 ml of distillated water to measure the alcohol content. The alcohol content was determined using a hydrostatic scale, at 20℃, using 100 ml of distillate obtained from a 200 ml sample distillation mixture using a small distiller.

Free sugars and organic acids

HPLC was performed to measure the free sugars on FBPE. A 1 ml FBPE sample was mixed with 4 ml of HPLC grade water to prepare the HPLC sample. Then, 2 ml of the diluted sample was filtered through a 0.45 μm Minipore PVDF filter (Schleicher & Schuell, BioScience, Germany) and Sep-Pak NH_2 column (Water Co., USA) for HPLC analysis. The injection volume was 20 μl. The sugar analysis was carried out on HPLC using a Polyamine II column. The 75% acetonitrile was eluted at a flow rate of 1 ml/min at 55℃. The various sugars were measured on an RI detector. In the HPLC analysis, sugars were detected approximately 5.8 to 11.9 min, depending on the structure of sugar. The free sugars were quantitated using standard curve analyzes with standard solutions of 1.0, 2.5, 5.0, 7.5, and 10 g/L.

HPLC was performed to determine the organic acids in the FBPE. A 1 ml aliquot of sample was mixed with 9 ml of HPLC grade water to prepare the HPLC sample. 2 ml of the diluted sample was filtered through a 0.45 μm Minipore PVDF filter for HPLC analysis. The injection volume was 20 μl. The organic acid analysis was carried out on an HPLC using a TSKgel ODS-100V column. The 0.1% $H_3PO_4$was eluted with a flow rate of 1 ml/min at 40℃. The various organic acids were measured at 210 nm with a UV detector. In the HPLC analysis, organic acids were detected approximately 3.1 to 15.4 min, depending on their structure. The organic acids were quantitated using standard curve analyzes with standard solutions of 10, 25, 50, 75, and 100 mg/L.

Recombinant DNA techniques

Standard procedures for recombinant DNA techniques were used as described by Sambrook and Russell (Sambrook and Russell, 2001).

Enrichment culture and DNA extraction

A 5 ml of FBPE was inoculated into 100 ml of MRS and PD broth with 0%, 20%, and 40% sucrose and enriched at 28℃ for 30±6 h and after the enriched samples were centrifuged at 13,000 × *g* for 5 min at 4℃. The obtained pellets (approximately 3 ml) were then subjected to DNA extraction using the G-spin Genomic DNA Purification Kit (iNtRON Biotechnology, Korea), as recommended by the supplier. The extracted DNA was then used as a template for the PCR amplification of the ribosomal RNA (rRNA) and after PCR products were performed the rRNA library construction and DNA sequence analysis.

PCR amplification of 16S and 26S rRNA genes

The extracted DNA was used as a template for PCR to amplify 16S and 26S rRNA genes. The bacterial-specific PCR primers 5′-CGGAGAGTTTGATCCTGG-3′ (1BF, forward primer) and 5′-TACGGGCTACCTTGTTACGAC-3′ (2BR, reverse primer) were used to amplify the 16S rRNA gene fragments (Cho and Seo, 2007) and the yeast-specific PCR primers 5′-ACCCGCTGAAYTTAAG CATAT-3′ (3YF/21 mer, forward primer based on the position of the Saccharomyces cerevisiae LSU rRNA) and 5′-CTCCTTGGTCG TGTTTCAAGACGG-3′ (3YR/25 mer, reverse primer) were used to amplify 26S rRNA gene fragments (Cho *et al*., 2009). The rRNAs were amplified by PCR using the metagenomic DNA and Super-Therm Taq DNA polymerase (JMR, UK). As indicated by the manufacturer's instructions, the PCR reaction mixture (50 μl) contained 1 μl of Taq polymerase (2.5 unit), 3 μl of each primer (1BF-2BR or 3YF-3YR, 10 pmol), 5 μl of reaction buffer, 15 mM MgCl2, 5 μl of 2 mM dNTP, 5 μl of template DNA, and 28 μl of sterilized water. Thirty cycles of denaturation at 94℃ for 30 sec, annealing at 50℃ for 30 sec, and extension at 72℃ for 90 sec or 60 sec were followed by a final incubation at 72℃ for 10 min. The anticipated 16S rRNA and 26S rRNA PCR product of approximately 1.5 kb and 0.6 kb, respectively, was isolated by agarose gel electrophoresis. The bacterial 16S rRNA and yeast 26S rRNA gene amplicons were purified using a PCR Purification Kit (iNtRON Biotechnology, Korea).

Ribosomal RNA library construction

Amplified bacterial or yeast rRNA partial genes were cloned in the pGEM-T easy vector in *E. coli* DH5α according to the manufacturer's instructions. Recombinant clones were picked randomly, and recombinant plasmids were extracted using the Plasmid DNA Purification Kit (iNtRON Biotechnology). Purified plasmids were checked for correct insert size via standard vector-targeted PCR and gel electrophoresis.

DNA sequencing and sequence analysis

Nucleotide sequencing was performed using the dideoxy chain-termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., USA) and an automated DNA sequencer (Applied Biosystems, USA). All of the reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project databases (http://rdp8.cme.msu.edu). Sequences were analyzed using the CHIMERA program (Maidak *et al*., 2000), to identify and exclude sequences arising from chimeric rRNA clones. The 16S and 26S rRNA sequence identity searches were performed using the BLSATN and PSI-BLAST tools on the NCBI web site (McGinnis and Madden, 2004), the sequences were aligned using the multiple sequence alignment program, CLUSTALW (Thompon *et al*., 1994). The phylogenetic analysis was performed by the neighbor-joining methods (Saitou and Nei, 1987), gaps and positions with ambiguities were excluded from the phylogenetic analysis. Bootstrap analysis was performed on data resampled 1,000 times using the DNAMAN analysis system (Lynnon Biosoft, Canada). Nucleotide sequences have been deposited in the GenBank database under the accession

Table 1. Characterization of physicochemical prosperities in the fermented beverage of plant extract

Component[a]	Fermented beverage of plant extract
pH	3.48 ± 0.12
Acidity (%, as lactic acid)	1.63 ± 0.02
Brix°	70.0 ± 0.15
Reducing sugar (g/L)	1,026 ± 1.13
Alcohol (%, v/v)	3.5 ± 0.02
Free sugar (g/L)	
Glucose	567.83 ± 0.11
Fructose	203.46 ± 0.09
Sucrose	ND[b]
Free organic acid (g/L)	
Oxalic acid	7.53 ± 0.01
Tartaric acid	93.68 ± 0.12
Malic acid	9.02 ± 0.02
Ascorbic acid	11.95 ± 0.02
Lactic acid	34.09 ± 0.10
Acetic acid	21.23 ± 0.08
Citric acid	29.46 ± 0.06
Sunnic acid	ND
Glutaric acid	ND

[a] Values indicate the mean's of three replications (n=3).
[b] ND, not detected

numbers JX441600-JX441613.

Results

Physicochemical prosperities of FBPE

The FBPE was collected at the Farming Cooperation Mario, in Goseong-gun, Korea. The FBPE was produced by the fermentation of a mixture of extracts from approximately 60 fruits, vegetables, sea algae, and medicinal herbs and was fermented for a three year periods. The FBPE had a pH of 3.48, 1.68% acidity, 70 °brix of soluble material, 1,026 g/L of reducing sugar, and 3.5% of ethyl alcohol. The major reducing sugars observed by HPLC analysis were glucose (567.83 g/L) and fructose (203.46 g/L). The organic acid contents of FPEB were tartaric acid (93.68 mg/L) > lactic acid (34.09 mg/L) > citric acid (29.46 mg/L) > acetic acid (21.23 mg/L) > ascorbic acid (11.95 mg/L) > malic acid (9.02 mg/L) > oxalic acid (7.53 mg/L) (Table 1).

Detection and cloning of rRNA genes

The microbial diversity of the FBPE was assessed in samples of enrichment cultures collected from three different enrichment cultures of sugar concentrations: MRS and PD broth with 0%, 20%, and 40% sucrose. PCR amplification of total DNA mixtures from three different FBPE samples with bacterial and yeast specific primers produced single or double amplification products of approximately 1.5 or 0.55 to 0.65 kb, respectively. The PCR products were isolated using PCR Purification Kit and cloned into the pGEM-T easy vector, which subsequently transformed into *E. coli* DH5α. A total of 261 clones were obtained from six libraries: MRS broth with 0% sucrose (0B), MRS broth with 20% sucrose (20B), MRS broth with 40% sucrose (40B), PD broth with 0% sucrose (0Y), PD broth with 20% sucrose (20Y), and PD broth with 40% sucrose (40Y).

Similarity with published sequences from an enrichment culture of FBPE

All clones from all six libraries were subjected to sequence analysis followed by online searches using two databases: GenBank, which implements the BLAST algorithm (McGnnis and Madden, 2004), and the RDP database, which implements the SIMILARITY_RANK program (Maidak *et al*., 2000) (Table 2). In general, if a sequence has greater than 98% similarity to the rRNA of a known microorganism, it is considered a member of that species.

All 135 clones in three bacterial libraries were identified as belonging to *Lactobacillus homohiochii* DSM20571 (133 clones) or *Lactobacillus fructivorans* DSM20203 (2 clones). All sequences in our libraries have greater than 99% similarity to a 16S rRNA of these known bacteria (Table 2).

Additionally, the 126 clones were divided into twelve groups based on their 26S rRNA sequences. Among them, seven species (groups MROJIY01, MROJIY03-04, MROJIY06, MROJIY09-10, and MROJIY12) were assigned to the *Candida* genus [*C. zeylanoides* TJY32 (45 clones), *C. zeylanoides* SY6X-2 (1 clones), *C. zeylanoides* TJY7b (1 clone), *C. lactis-condensi* (48 clones), *C. parapsilosis* 25N-2B (4 clones), *C. parapsilosis* 8.6 (4 clones), and *C. parapsilosis* KBP4100 (1 clone)], two species (groups MROJIY02 and MROJIY07) to the *Pichia* genus [*P. farinosa* IMAU:Y1021 (8 clones) and *P. guilliermondii* (7 clones)], two species (groups MROJIY05 and MROJIY11) to the *Zygosaccharomyces* genus [*Z. baillii* ATCC MYA-4549 (4 clones) and *Z. baillii* E (2 clones)], and only one species (group MROJIY08) to the *Kazachstania* genus [*K. exigua* CBS 379 (1 clone)]. The 121 clones (96.8%) in our libraries have greater than 98% similarity to a 26S rRNA of the known yeast. In addition, approximately 3.2% (6 clones) of the sequences have a similarity level with database sequences in the range of 88–97% (Table 2).

Distributions of lactic acid bacteria and yeast from an enriched culture of FBPE

The distribution of LAB species from FBPE are shown in Table 3. *L. homohiochii* was the predominant species in the all enrichment culture samples of FBPE: 100% of the species in the 0B (0% sugar) and 40B (40% sugar) libraries and 95.6% of the species in the 20B library (20% sugar). *L. fructivorans* were also detected in the 20B library. The distribution of yeast species from FBPE is shown in Table 3. The predominant species in samples of enriched culture of FBPE collected from three different sugar concentrations were: *C. zeylanoides* (19 clones, 45.2%) in the 0Y

Table 2. Similarity values of 16S and 26S rRNA sequences retrieved from the fermented beverage of plant extract

Group	Accession number	No. of clones	Phylum	Nearest relative (Accession No.)	Similarity[a] (%)
Bacteria		135			
MROJIB01	JX441600	133	*Firmicutes*	*Lactobacillus homohiochii* DSM20571 (AM113780)	**99**[b]
MROJIB02	JX441601	2	*Firmicutes*	*Lactobacillus fructivorans* DSM20203 (NR036789)	**99**
Yeast		126			
MROJIY01	JX441602	45	*Ascomycota*	*Candida zeylanoides* TJY32 (EU250065)	88-**100**
MROJIY02	JX441603	8	*Ascomycota*	*Pichia farinosa* IMAU:Y1021 (GU138424)	87-**100**
MROJIY03	JX441604	48	*Ascomycota*	*Candida lactis-condensi* (U45724)	**99-100**
MROJIY04	JX441605	4	*Ascomycota*	*Candida parapsilosis* 25N-2B (AB506708)	**99-100**
MROJIY05	JX441606	4	*Ascomycota*	*Zygosaccharomyces bailii* ATCC MYA-4549 (FJ914902)	88-**99**
MROJIY06	JX441607	4	*Ascomycota*	*Candida parapsilosis* 8.6 (EU605804)	**97-100**
MROJIY07	JX441608	7	*Ascomycota*	*Pichia guilliermondii* EQ (EU177574)	**100**
MROJIY08	JX441609	1	*Ascomycota*	*Kazachstania exigua* CBS 379 (EU982210)	**99**
MROJIY09	JX441610	1	*Ascomycota*	*Candida zeylanoides* SY6X-2 (EU285537)	**98**
MROJIY10	JX441611	1	*Ascomycota*	*Candida parapsilosis* KBP4100 (FR774537)	**98**
MROJIY11	JX441612	2	*Ascomycota*	*Zygosaccharomyces bailii* E (EU441913)	**99**
MROJIY12	JX441613	1	*Ascomycota*	*Candida zeylanoides* TJY7b (EU327107)	**99**

[a] Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence is given.
[b] Database sequences with > 97% similarity are shown in bold.

library (0% sugar), *C. lactis-condensi* (15 clones, 35.7%) and *C. zeylanoides* (15 clones, 35.7%) in the 20Y library (10% sugar), and *C. lactis-condensi* (16 clones, 38.1%) in the 40Y library (20% sugar). *C. lactis-condensi* (17 clones, 40.5%), *P. farinosa* (3 clones, 7.1%), *C. parapsilosis* (2 clones, 4.8%), and *Z. bailii* (1 clones, 2.4%) were also detected in the 0Y library; *P. guilliermondii* (6 clones, 14.3%), *P. farinosa* (3 clones, 7.1%), *C. parapsilosis* (2 clones, 4.8%), and *K. exigua* (1 clones, 2.4%) were found in the 20Y library; and *C. zeylanoides* (13 clones, 30.9%), *C. parapsilosis* (5 clones, 11.9%), *Z. bailii* (5 clones, 11.9%), *P. farinosa* (2 clones, 4.8%), and *P. guilliermondii* (1 clones, 2.4%) were identified in the 40Y library. *C. lactis-condensi*,

Table 3. Lactic acid bacterial and yeast distributions of the six libraries from enrichment cultures under various growth conditions[a]

Genus species	Number of clones / Percentage (%)			Number of total clones
	Sugar concentrations (%, w/v)			
	0	20	40	
Lactobacillus				
L. homohiochii	45 / 100	43 / 95.6	45 / 100	133
L. fructivorans		2 / 4.4		2
Number of total clones	45 / 100	45 / 100	45 / 100	135
Candida				
C. lactis-condensi	17 / 40.5	15 / 35.7	16 / 38.1	48
C. parapsilosis	2 / 4.8	2 / 4.8	5 / 11.9	9
C. zeylanoides	19 / 45.2	15 / 35.7	13 / 30.9	47
Kazachstania				
K. exigua		1 / 2.4		1
Pichia				
P. farinosa	3 / 7.1	3 / 7.1	2 / 4.8	8
P. guilliermondii		6 / 14.3	1 / 2.4	7
Zygosaccharomyces				
Z. bailii	1 / 2.4		5 / 11.9	6
Number of total clones	42 / 100	42 / 100	42 / 100	126

[a] The 5 ml of the fermented beverage of plant extract was inoculated into 100 ml of MRS and PD broths with 0%, 10%, and 20% sucrose and enriched at 28℃ for 30 ± 6 h.

C. parapsilosis, *C. lactis-condensi*, and *P. farinosa* were found in three libraries. *K. exigua* was detected in the 20Y library.

Discussion

The LAB and yeast composition of FBPE were examined by PCR-based analysis of bacterial 16S rRNA and yeast 26S rRNA genes, respectively. In this study, PCR-based analysis under various culture condition was carried out after enrichment culture due to the limitations of PCR-based analysis (Farrelly *et al*., 1995; Zhang *et al*., 2011). PCR-based methodologies are subject to certain limitations such as the PCR amplification error and formation of chimera (Cho *et al.*, 2008). Also, Lee and Kim (2000) suggested that PCR amplification cannot be strictly co-related with the ratio of target DNA to total DNA. As a result, some minor population may have been missed in our present study. The FBPE system environments represent extreme osmotic pressure. Extreme environments are interstation gore the study of microbial diversity, the identification of novel microorganisms, and the understanding of the functioning of their ecosystems. The microbial diversity in FBPE has not yet been reported.

In the present study, an extract from a blend of 60 fruits, vegetables, sea algae, and medicinal herbs was fermented to produce a new beverage. This sample was extracted using sucrose-osmotic pressure in a pilot stainless steel tank for 5−10 days and fermented by lactic acid bacteria and yeast for three years. In our sample, the glucose and fructose contents were 567.83 and 203.46 g/L, respectively (Table 1). Okada *et al*. (2006) previously reported that the FBPE contained high levels of saccharides, estimated between 550 and 590 g/L, mainly glucose and fructose. In addition, Okada and colleagues previously reported the presence of small amounts of oligosaccharide, such as laminaribiose, maltose, and β-D-fructopyranoside. The extract in that study was obtained using sucrose-osmotic pressure in cider barrel for one week and fermented by LAB (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.) for 180 days (Okada *et al*., 2006, 2009, 2010).

In the LAB analysis, LABs all closely related to *L. homohiochii* and *L. fructivorans* were found in the three libraries (Tables 2 and 3). *L. homohiochii* was found to be the predominant yeast in FBPE samples. *L. homohiochii* was previously isolated from the fermented foods sake (Kondo and Ikeda, 2000), and kimchi (So and Cho, 1999). Both *L. homohiochii* and *L. fructivorans*, are considered “hiochi bacteria”, which is a general term for lactobacilli that can grow in beverages such as sake and can cause brewed beverages to spoil. Hiochi bacteria are further subdivided as alcoholophilic and alcohol-tolerant bacteria such as *L. fructivorans*, *L. fermentum*, *L. acidophilus*, *L. heterohiochi*, *L. homohiochi*, and *L. tricodes*. Additionally, hiochi bacteria are classified into homofermentative and heterofermentative types based on the products of lactic acid fermentation. *L. homohiochi* is the former type of true hiochi-bacilli, while *L. fructivorans* is the latter type (Kondo and Ikeda, 2000; Ohbuchi *et al*., 2001; Wada and Mizoguchi, 2007). Hiochi bacteria, such as *L. homohiochi* and *L. fructivorans*, are present in the FBPE, which is usually made from the numerous fresh fruits, vegetables, algae, and medicinal herbs. Because the FBPE are fermented in a natural environment, several microorganisms may be involved in its colonization. Therefore, FBPE fermentation begin as an open ecosystem and after it becomes closed during the fermentation process, each batch of fermented FBPE develops a unique microbial population depending on the length of the fermentation period. Furthermore, hiochi bacteria can be grow if the FPEB is ripened or alcohol fermented. In the future, lactic acid bacterial diversity during the manufacture, ripening, or fermentation of FBPE will be investigated.

In the yeast analysis, yeasts most closely related to *C. zeylanoides* and *C. lactis-condensi* were found in the three libraries (Tables 2 and 3). *C. zeylanoides* had previously been isolated and identified in the fermented foods goat’s milk (Fadda *et al*., 2010), cheese (Romano *et al*., 1996), and sausage (Cocolin *et al*., 2006). *C. zeylanoides* was the most frequently occurring species followed by different *Basidiomycetous* species. Some biochemical characteristics of technological interest were investigated in the isolated strains, and a predominance of lipolytic yeast species was found (Fadda *et al*., 2010). *C. lactis-condensi* and *Z. baillii* had been previously isolated and identified in two fermented foods, traditional balsamic vinegar (Solieri *et al*., 2006; Solieri and Giudici, 2008), and vino cotto (high-sugar grape must) (Tofalo *et al*., 2009). *C. lactis-condensi* and *Z. baillii* were appeared at higher concentrations in the 40Y library (sugar concentration = 40%) than in other two libraries (Table 3). Many yeast species preferentially ferment fructose (fructophilic metabolism) and leave glucose unfermented. *C. lactis-condensi* has been found to have the highest fructophilic phenotype in sugar-fermented food because they leave the initial glucose amount unchanged and completely consume the fructose. *Z. baillii* also showed a fructophilic metabolism (Solieri and Giudici, 2008). The fructophilic behavior of *Z. baillii* is dependent on a fructose-specific high capacity system, while the glucose is transported by a lower capacity system that is, partially inactivated by fructose (Pina *et al*., 2004). This strain is known to possess unusual physiological characteristics, such as resistance to weak-acid preservatives and extreme osmotolerance up to 4 M glucose (72% w/v) (Martorell *et al*., 2007). *Pichia* species have been found in fermented foods such as table olives (Tofalo *et al*., 2012), sourdough (Zhang *et al*., 2011), and wine (Li *et al*., 2010).

During fermentation, the beneficial role of yeasts lies in the production of desirable volatile compounds and metabolites that improve the organoleptic properties, enhancement of the growth

Table 4. Overview of relationship between phylum and family analysis results each of library

Kingdom Phylum Family	Number of clones / Percentage (%)			Number of total clones
	Sugar concentrations (%, w/v)			
	0	20	40	
Bacteria[a]				
Firmicutes				
Lactobacillaceae	45 / 100	45 / 100	45 / 100	135 / 100
Yeast[b]				
Ascomycota				
Saccharomycetaceae	39 / 92.9	33 / 78.6	39 / 92.9	111 / 88.1
Debaryomycetacease	3 / 7.1	9 /21.4	3 / 7.1	15 / 11.9

[a] The 5 ml of the fermented beverage of plant extract was inoculated into 100 ml of MRS broth with 0%, 20%, and 40% sucrose and enriched at 28℃ for 30 ± 6 h.
[b] The 5 ml of the fermented beverage of plant extract was inoculated into 100 mL of PD broth with 0%, 20%, and 40% sucrose and enriched at 28℃ for 30 ± 6 h.

of LAB, killer activity, and the biodegradation of phenolic compounds (Nisiotou *et al*., 2009). On the other hand, yeasts may cause gas pocket formation due to excessive CO_2 production at the early stage of fermentation (Lamzira *et al*., 2005), and softening of the plant tissue (Hernandez *et al*., 2007). Finally, the activity of yeast at the packing stage may be detrimental, resulting in package bulging caused by CO_2 accumulation, clouding of the brines, production of off-flavors and odors, and resistance to preservatives (Turantas *et al*., 1999). In the future, the diversity of yeast during the manufacture, ripening, or fermentation of FBPE should be investigated.

This study was performed to investigate the microbial diversity in the FBPE by analyzing bacterial 16S rRNA and yeast 26S rRNA sequences. All sequences derived from the three bacterial libraries (0B, 20B, and 40B) were related to those of the *Lactobacillaceae* family, and most of the sequences derived from the three yeast libraries (0Y, 20Y, and 40Y) were related the *Saccharomycetaceae* family (Table 4). It is also assumed that the one sample used in the present study is not sufficient to cover the diversity of the LAB and yeast in FBPE. To characterize the biodiversity of LAB and yeast in FBPE, it well is necessary to compare the microbial diversity in various FBPE samples collected from different fermentation periods or many products. The scope of the functional roles and the extent of LAB and yeast diversity have yet to be understood, given that most LAB and yeast in FBPE remain undescribed. The PCR primers described in this report provided unique tool to further characterize this important group of organisms (Borneman and Hartin, 2000). Considering that the variation in each position amounts to 5%, when prokaryotic and eukaryotic nuclear large subunit (LSU) rRNA were amplified as reference and using various universal primers (Cho and Seo, 2007; Cho *et al*., 2009), the PCR primer used to amplify 16S and 26S rRNA used in this study was not sufficient to cover most phyla of yeast in FBPE. A more specific set of phylogenetic primers for the microorganism will be used in future studies to more fully characterize the microbial diversity of FBPE.

In conclusions, we have shown that enrichment culture and culture-independent methods can be successfully applied to explore the microbial diversity in FBPE. The results revealed that the LAB and yeast communities in FBPE were different than we expected. They provide novel insights into the LAB and yeast communities in FBPE. The data presented in this study may provide a useful frame of reference for further analyses of microbial population dynamics in FBPE, flavor development, and control of the fermentation process. Furthermore, starters for culture are not generally used in FBPE production at present, because operators are reluctant to introduce them in a rigorously traditional field. However, the development of culture starters could improve the quality of FBPE. They may decrease the frequency of "stuck fermentations" and mold growth, successfully increase the growth of LAB and positively affect the economical yield of FBPE.

적 요

식물추출발효음료(fermented beverage of plant extract, FBPE)는 과일류, 야채류, 약초류, 및 해조류를 설탕에 의한 당장법으로 발효시킨 액상발효음료이다. 본 연구에서는 FBPE의 이화학적 특성과 16S 및 26S rRNA 염기서열을 이용하여 집식배양된 FBPE의 미생물 다양성을 조사하였다. FBPE의 pH는 3.48, 산도는 1.68%, 당도는 70°, 환원당은 1,026 g/L 및 알코올은 3.5%이었다. 유리당과 유기산은 glucose (567.83 g/L)와 tartaric acid (936.8 mg/L)로 나타났다. *Lactobacillus homohiochii*는 모든 집식배양 시료에서 우점종이었고 *L. fructivorans*는 20B 라이브러리에서만 나타났다. 세 가지의 다른 당 농도에서 집식배양된 FPEB의 우점종은 0Y 라이브러리(설탕농도 0%)에서는 *Candida zeylanides* (45.2%), 20Y 라이브러리(설탕농도 20%)에서는 *C. lactis-condensi* (35.7%)와 *C. zeylanoides* (35.7%) 및 40Y 라이브러리(설탕농도 40%)에서는 *C. lactis-condensi* (38.1%)이었다. 이외에 0Y 라이브러리에서는 *C. lactis-condensi* (40.5%), *Pichia farinosa* (7.1%), *C. parapsilosis* (4.8%) 및 *Zygosaccharomyces bailii* (2.4%)가 나타났으며, 20Y

라이브러리에서는 *P. guilliermondii* (14.3%), *Pichia farinosa* (7.1%), *C. parapsilosis* (4.8%) 및 *Kazachstania exigua* (2.4%)로 나타났고 40Y 라이브러리에서는 *C. zeylanoides* (30.9%), *C. parapsilosis* (11.9%), *Z. bailii* (11.9%), *Pichia farinosa* (4.8%), *P. guilliermondii* (2.4%)이 확인되었다. 이 결과는 앞으로 FBPE의 미생물 변화 연구에 아주 유용한 자료로 제공할 수 있다.

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