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Microbial Dynamics of Commercial *Makgeolli* Depending on the Storage Temperature

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Market fresh *makgeolli* was stored at different temperatures of 4°C and 25°C to assess the change of the microbial diversity according to the storage temperature and period. Yeast counts increased until day 3 of storage and decreased thereafter. General and lactic acid bacterial counts continuously increased during storage. The data indicated that the control of growth of microorganisms, particularly general bacteria and lactic acid bacteria (LAB), is essential. Total acid levels started to decrease in the *makgeolli* stored at 4°C, and increased from day 6 of storage in the *makgeolli* stored at 25°C. The increase of total acid in the non-refrigerated condition greatly affected the quality of *makgeolli*. In both the fresh *makgeolli* samples stored at 4°C and 25°C, yeast (*Saccharomyces cerevisiae*) and molds (*Aspergillus tubingensis, Candida glaebosa,* and *Aspergillus niger*) were noted. Denaturing gradient gel electrophoresis (DGGE) band patterns were almost constant regardless of the storage period. As for bacteria, *Lactobacillus crustorum, L. brevis*, and *Microlaena stipoides* were found in the *makgeolli* stored at 4°C, and *L. crustorum, Lactobacillus* sp., *L. plantarum, L. brevis, L. rhamnosus*, and *L. similis* were found in the *makgeolli* stored at 25°C. In particular, in the *makgeolli* stored at 25°C, *L. crustorum* and *L. plantarum* presented dark bands and were identified as the primary microorganisms that affected spoilage of fresh *makgeolli*.

Keywords: Microbial dynamics, *makgeolli*, denaturing gradient gel electrophoresis, storage

Makgeolli, which is also called *takju*, is a traditional Korean alcoholic beverage that is made by steaming glutinous and non-glutinous rice, barley, and flour, among other ingredients. The mixture is later fermented with *nuruk* and water. *Makgeolli* is distinguished from other alcoholic beverage in terms of its nutritional content, being enriched in vitamin B group compounds, essential amino acids such as lysine, leucine, and glutathione, as well as protein, sugar, and live yeast [7]. Recently, the consumption of *makgeolli* in Korea and Japan has risen dramatically. The liquor market share of domestic *makgeolli* has not dropped below 5% in the past five years, and in fact has grown in market share, from 7.8% in 2009 to 12% in the first quarter of 2011.

Makgeolli is cheap liquor that is traditionally drunk by lower socioeconomic classes. It is regarded as representative Korean liquor. *Yakju,* which is the traditional beverage used in Korea to offer toasts, is made by filtering *makgeolli* to produce a clear rice wine. *Makgeolli* further gained popularity as the emphasis towards health and well-being. This soaring popularity has prompted more studies on the functionality of *makgeolli*, including the potential anti-inflammatory effects of malt extract [3], anticancer effect [14], and the effect of food additives on improving quality [2, 6, 12]. Diverse molds, bacteria, and yeasts adhere to wheat or oats in *makgeolli* during the fermentation and ripening processes, consequently affecting the fermentation of *makgeolli*. These microorganisms are not eliminated even in the mash screening process; their continued presence deteriorates the quality of *makgeolli* according to the storage temperature or period [15, 20]. However, there have been relatively few studies on the microorganisms that contribute to spoilage in *makgeolli*.

The DGGE process uses the polymerase chain reaction (PCR) of DNA and determines diverse species using the amplified PCR product [8]. DGGE enables the isolation depending on the melting temperature (Tm) according to the base composition of the DNA fragment.

This study analyzed the change in the physiochemical characteristics of market fresh *makgeolli* during storage.

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Simultaneously, the microorganisms isolated from this *makgeolli* collected by time zone were analyzed by DGGE to identify those microorganisms that affected *makgeolli* quality.

Materials and Methods

Materials

This test used fresh *makgeolli* (*Jangsoo-makgeolli,* Seoul, Korea) obtained from the market. Samples were stored at 4°C and 25°C, to allow examination of the change in microorganisms according to the storage temperature and period. Samples were taken from the original commercial bottles at 3-day intervals for a period of 12 days.

Enumeration of Microorganisms

To enumerate microorganisms during the storage of fresh *makgeolli*, 1 ml of each collected sample was uniformly mixed with saline solution at every stage. Yeasts were enumerated by spreading 100 µl of sample solution on potato dextrose agar [0.4% (w/v) potato starch, 2% (w/v) dextrose; Difco, MD, USA] with filter-sterilized chloramphenicol [0.1% (v/v); Sigma-Aldrich, MO, USA] and cultured at 30°C for 48 h. LAB were enumerated after each sample was anaerobically cultured using the MRS medium (Difco, MD, USA) with amphotericin solution [1% (v/v); Sigma-Aldrich, MO, USA] at 30°C for 48 h. The viable cells were enumerated after each sample was aerobically cultured using the plate count agar medium [0.5% (w/v) pancreatic digest of casein, 0.1% (w/v) dextrose, 0.25% (w/v) yeast extract; Difco, MD, USA] with amphotericin solution [1% (v/v); Sigma-Aldrich, MO, USA] at 30°C for 24 h. The colony counts were recorded as log colony forming units per milliliter (Log CFU/ml).

DNA Extraction

The collected *makgeolli* pellet was ground using liquid nitrogen and 30 ml of STES buffer [0.5 M NaCl, 0.2 M Tris-HCl (pH 7.6), 0.01 M EDTA, 1% (w/v) sodium dodecyl sulfate (SDS)] was added. The mixture was avidly stirred and centrifuged at 12,000 rpm for 10 min, and the supernatant was collected. The same quantity of phenol:chloroform:isoamyl alcohol (24:25:1) was added to the substance and centrifuged at 4,000 rpm for 10 min. The same quantity of chloroform was added to its supernatant and centrifugation was similarly conducted. The resulting supernatant was collected and treated with 0.1% (v/v) RNase A solution (Intron, Seongnam, Korea) at 37°C for 1h and additionally refined using a G-spin bacterial genomic DNA extraction kit (Intron, Seongnam, Korea). The product was used as the sample for the molecular biological analysis.

DNA Amplification by Polymerase Chain Reaction

PCR was performed in a C1000 DNA thermal cycler (Bio-Rad, CA, USA). This process used the DNA extracted from fresh *makgeolli* as its template. Fungal 18S rRNA was first PCR-amplified targeting the 18S and 28S regions with primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4R (5'-TCC TCC GCT TAT TGA TAT GC-3'), followed by a second PCR amplified with ITS1F (with GC-clamp) 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA-3' and ITS2R 5'-GCT GCG TTC TTC ATC GAT GC-3' [16–18]. This product was used for DGGE. The Maxime PCR PreMix Kit (Intron, Seongnam, Korea) was used for the PCR mixture. PCR comprised a pre-denaturation step at 95°C for 2 min, denaturation at 95°C for 1 min, annealing at 50°C, and extension at 72°C for 1 min repeated in 34 cycles. The product underwent post-extension at 72°C for 10 min. Prokaryotic 16S rRNA was PCR-amplified targeting the V3 region using primers B357F (with GC-clamp) 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TACG GGA GGC AGC AG-3' and U519R 5'-ACC GCG GCT GCT GGC AC-3'. The pre-denaturation process at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 58°C, and extension at 72°C for 30 s was repeated for 34 cycles. The product underwent post-extension at 72°C for 10 min. The amplified PCR products were analyzed on 1.5% agarose gel and their sizes examined using a 1 kb DNA ladder (Bioneer, Daejeon, Korea). Bacteria and yeasts (including molds) were magnified to the sizes of 200 bp and 500 bp, respectively.

DGGE Analysis

The amplified PCR products were analyzed by DGGE using the DCode system (Bio-Rad, CA, USA). In the denaturing gradient gel, urea (Sigma-Aldrich, MO, USA) and formamide (Bioneer, Daejeon, Korea) denaturants were added to 10% polyacrylamide (37.5:1 ratio of acrylamide:bisacrylamide; Bioneer, Daejeon, Korea) at a concentration gradient of 20%–60%. Twenty microliters of the amplified PCR products was loaded onto the gel, and electrophoresis was conducted in 0.5× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) at 60°C and 20 V for 10 min, and then at 60°C and 80 V for 15 h. Following electrophoresis, the gel was dyed with SYBR Green I (Bio-Rad, CA, USA). The resolved band was revealed using the Gel Doc XR$^+$ Molecular Imager (Bio-Rad, CA, USA) and excised from the gel.

Base Sequence Analysis

To collect the DNA fragments from different positions in the denaturing gradient gel, each band was selected and excised. Fifty microliters of deionized water was added to each gel slice and left overnight at 4°C prior to centrifugation at 6,300 ×*g* for 5 min to obtain the supernatant. With the DNA from each band as the template, PCR was conducted using the ITS1F and ITS2R primers for yeasts and molds, and the B357F and U519R primers for bacteria. After PCR, the base sequence analysis was contracted to Macrogen (Seoul, Korea). Similarity searches for nucleotide sequences were performed using the Web-based BLAST algorithm of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

Chemical and Sensory Analyses of *Makgeolli*

The pH was measured with a model D-51 pH meter (HORIBA, Kyoto, Japan). The concentration of soluble solids was measured with a Pocket PAL-1 hand-held refractometer (ATAGO, Tokyo, Japan) and recorded in Brix units (% sucrose). Total acid was measured as citric acid concentration and determined by adding 2–3 drops of phenolphthalein indicator to 10 ml of sample and titrating with 0.1 N NaOH until the solution turned light green. Phthalic acid (Sigma-Aldrich, MO, USA) was used as the standard [9]. The sensory evaluation was conducted by 12 judges (eight female, four male) recruited from the Korea Food Research Institute. Overall acceptance of the *makgeolli* was measured on a nine-point hedonic scale (1 = dislike extremely, 9 = like extremely).

Table 1. Microbial cell counts of commercial fresh *makgeolli* depending on the storage temperature for 12 days.

Days	Microbial cell counts (log CFU/ml)****					
	4°C			25°C		
	Yeast	Aerobic bacteria	Lactic acid bacteria	Yeast	Aerobic bacteria	Lactic acid bacteria
0	7.76 ± 0.02^{a}	5.70 ± 0.07^{c}	5.59 ± 0.03^{d}	7.75 ± 0.02^{a}	5.77 ± 0.12^{c}	5.62 ± 0.12^{d}
3	7.81 ± 0.08^{a}	5.77 ± 0.02^{c}	5.73 ± 0.12^{d}	7.63 ± 0.03^{a}	5.71 ± 0.08^{c}	5.81 ± 0.13^{c}
6	6.91 ± 0.05^{b}	6.30 ± 0.06^{b}	6.27 ± 0.14^{c}	6.82 ± 0.03^{b}	5.70 ± 0.05^{c}	6.88 ± 0.07^{b}
9	6.81 ± 0.02^{c}	6.70 ± 0.06^{a}	6.71 ± 0.06^{b}	6.38 ± 0.05^{c}	6.06 ± 0.25^{b}	6.85 ± 0.02^{b}
12	4.78 ± 0.06^{d}	6.71 ± 0.06^{a}	7.84 ± 0.02^{a}	5.72 ± 0.15^{d}	6.62 ± 0.12^{a}	7.63 ± 0.12^{a}

Results are presented as mean ± SD (n = 3).
****P < 0.0001.
Means with different letters across the line are significantly different at the 5% level by Duncan's multiple range test.

Statistical Analysis

The mean separation of the experimental parameters was determined by the analysis of variance (ANOVA). The statistical analysis was performed using SAS for Windows ver. 7.2 (Statistical Analysis Systems Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Microbial Changes of *Makgeolli*

For *makgeolli* stored at 4°C, the yeast counts increased from the initial 7.76 log CFU/ml to 7.81 log CFU/ml by day 3, and then decreased to 6.81 log CFU/ml by day 9 ($p<0.05$). The bacterial counts increased from the initial 5.7 log CFU/ml to 6.71 log CFU/ml by day 12 ($p<0.05$). Lactic acid bacterial counts increased from the initial 5.59 log CFU/ml to 7.84 log CFU/ml for the 12th day of storage ($p<0.05$). For storage of market fresh *makgeolli* stored at 25°C, yeast counts decreased from the initial 7.75 log CFU/ml to 6.82 log CFU/ml on day 6, and to 5.72 log CFU/ml on day 12 ($p<0.05$). The bacterial counts increased from the initial 5.77 log CFU/ml to 6.62 log CFU/ml by day 12 ($p<0.05$). Lactic acid bacterial counts increased from the initial 5.62 log CFU/ml to 7.63 log CFU/ml for the 12th day of storage ($p<0.05$) (Table 1). In a previous study [21], the bacterial counts increased from the initial 10^6 CFU/ml to 10^7 CFU/ml on day 5 of fermentation, and yeast counts increased to 10^7 CFU/ml on day 5 of fermentation and decreased to 10^6 CFU/ml on day 9, which almost exactly coincided with the present findings. In contrast, Seo *et al*. [13] reported that bacterial counts during fermentation increased to 10^9 CFU/ml with a subsequent decrease to 10^8 CFU/ml on day 3. In the same study, the yeast counts increased from the initial 10^5 CFU/ml to 10^8 CFU/ml, whereas the lactic acid bacterial counts were initially similar to the bacterial counts, but gradually decreased during fermentation. These results differed from those of the present study. This discrepancy may be attributed to differences in *makgeolli* brewing methods, malts and yeasts, and storage temperatures. Although there are differences in the bacterial counts and the trend of increase/decrease according to the fermentation period, studies were condunted to control the growth of microorganisms, especially the general bacteria and LAB. Considering the increase in the bacterial counts, particularly the accumulated lactic acid due to the continuous growth of LAB and the resulting acid production, this should have the effect of lengthening the effective storage period of *makgeolli*.

Chemical Changes of *Makgeolli*

Table 2 summarizes the changes in pH, sugar content, total acid, and the overall preference according to the storage temperature and time of market fresh *makgeolli*. For *makgeolli* stored at 4°C, the pH constantly increased from the initial 3.39 to 4.16 by day 12 ($p<0.05$). For *makgeolli* stored at 25°C, pH increased from the initial 3.39 to 4.19 on day 9, and thereafter decreased to 4.12 on day 12 ($p<0.05$). The sugar content increased from the initial 3.9 to 4.2% on day 6, and then decreased on day 9, to 2.9% on day 12 for *makgeolli* stored at 4°C ($p<0.05$). For *makgeolli* stored at 25°C, sugar content was 4.3% on day 9, followed by a decrease to 4.0% on day 12 ($p<0.05$). In a prior study that examined the change in sugar content of chitosan-supplemented *makgeolli* during storage [5], the sugar content also increased until day 6 and decreased thereafter. Another study reported that the sugar content decreased in the late stage of fermentation owing to the decomposition of the starch in the raw material to sugar *via* diastasic action during fermentation, with the sugar being used as the source of nutrition for the microorganisms or as a fermentation substrate [10]. The total acid increased from the initial 0.20 to 0.22% on day 9 and decreased to 0.16% on day 12 in the *makgeolli* stored at 4°C, but increased to 0.31% on day 6 in *makgeolli* stored at 25°C ($p<0.05$). The pH generally decreases because of the activation of LAB and yeast during the *makgeolli* storage. Lee and Shim [7] report that the pH increased until day 10 and decreased beginning around day 20. In addition, Kim *et al*. [4] reported that in the *takju* and *yakju* fermentation process,

Table 2. Chemical contents and overall preference of commercial fresh *makgeolli* depending on the storage temperature.

Days	4°C				25°C			
	pH****	Soluble solid (%, sucrose)**	Total acid (%, acetic acid)	Overall acceptance*	pH****	Soluble solid (%, sucrose)***	Total acid (%, acetic acid)**	Overall Acceptance***
0	3.39 ± 0.02^{d}	3.9 ± 0.14^{a}	0.20 ± 0.01^{a}	6.3 ± 1.11^{a}	3.39 ± 0.03^{d}	3.9 ± 0.07^{d}	0.20 ± 0.01^{b}	6.3 ± 1.11^{a}
3	3.41 ± 0.01^{d}	3.9 ± 0.14^{a}	0.20 ± 0.01^{a}	5.9 ± 0.38^{ab}	3.94 ± 0.01^{c}	3.8 ± 0.14^{e}	0.19 ± 0.01^{b}	3.9 ± 0.90^{b}
6	3.54 ± 0.01^{c}	4.2 ± 0.14^{a}	0.22 ± 0.01^{a}	5.6 ± 0.79^{abc}	3.97 ± 0.01^{c}	4.2 ± 0.14^{b}	0.31 ± 0.03^{a}	3.0 ± 0.82^{c}
9	3.98 ± 0.03^{b}	4.1 ± 0.0^{a}	0.22 ± 0.03^{a}	5.3 ± 1.11^{bc}	4.19 ± 0.01^{a}	4.3 ± 0.14^{a}	0.31 ± 0.01^{a}	2.3 ± 0.49^{cd}
12	4.16 ± 0.07^{a}	2.9 ± 0.01^{b}	0.16 ± 0.03^{a}	5.0 ± 1.15^{c}	4.12 ± 0.03^{b}	4.0 ± 0.14^{c}	0.31 ± 0.00^{a}	2.0 ± 0.58^{d}

Results are presented as mean ± SD (n = 3).
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Means with different letters across the line are significantly different at the 5% level by Duncan's multiple range test.

the pH decreases and titratable acidity increases as a result of growth of LAB in the early fermentation stage influencing the changes in general bacteria, LAB, and yeast in the next fermentation stage. The overall preference significantly decreased from an initial 6.3 point to 5 point on day 12 in *makgeolli* stored at 4°C ($p<0.05$). *Makgeolli* stored at 25°C scored a significantly low 3.9 point beginning at day 3, when LAB started to grow, and further decreased to 2 points on day 12 ($p<0.05$). By that time, it was unacceptable to drink. The results support the view that the quality of *makgeolli* is greatly affected by the storage temperature and that temperature control is essential to address the problems occurring from storage.

Denaturing Gradient Gel Electrophoresis

Diverse bands were identified in the DGGE profiles according to the storage period of fresh *makgeolli*. A total of 18 different bands were isolated for bacteria and 11 for yeasts (including molds). Tables 3 and 4 display the diversity of microorganism groups in market fresh *makgeolli* based on their DGGE profiles. The yeasts identified from bands No. 3, 5, and 11 in *makgeolli* stored at 4°C and 25°C were all *Saccharomyces cerevisiae.* As for molds, *Candida glaebosa* was the main band in No. 2, 4, 6, and 8; *Aspergillus tubingensis* in No. 1 band; *Aspergillus niger* in No. 10 band; and *Gibberella zeae* in No. 7 band. The pattern change in the DGGE band was almost constant regardless of the storage period (Fig. 1B). Meanwhile, the bacteria showed different changes in market fresh *makgeolli* samples stored at 4°C and 25°C (Fig. 1A). In the *makgeolli* stored at 4°C, *Microlaena stipoides* comprised the No. 9 band, and uncultured bacteria made up bands 3, 10, 11, and 17 during the storage. In *makgeolli* stored at 25°C, *Lactobacillus plantarum* (No. 5 band) and *L. crustorum*

Table 3. Identification of bacteria in the *makgeolli* samples by PCR-DGGE.

No.	Nearest neighbor strain	Similarity (%)	Acession No.
1	*Lactobacillus crustorum* IMAU:V3002	95	GU138496
2	*Lactobacillus* sp. EMML 3041	95	HQ389549
3	Uncultured compost bacterium, clone FS1368	97	FN667290
4	*Lactobacillus plantarum* clone WWC_C3ALM065	96	GU430806
5	*Lactobacillus plantarum* strain C8-1	93	FJ378889
6	*Lactobacillus* sp. EMML 3041	98	HQ389549
7	*Lactobacillus brevis* strain HDRS2	98	AY974809
8	*Lactobacillus crustorum* gene	97	AB626073
9	*Microlaena stipoides* chloroplast	96	GU592211
10	Uncultured bacteria, clone 380	95	FN776641
11	Uncultured bacterium clone GB7N87002C5BJJ	100	HM741099
12	Uncultured bacterium isolate DGGE gel band B16	93	GU301232
13	*Lactobacillus rhamnosus* clone WWC_C3AKM079	95	GU429381
14	*Lactobacillus rhamnosus* strain V92	100	JF444753
15	*Lactobacillus rhamnosus* clone WWC_C4AKM014a	91	GU425735
16	*Lactobacillus rhamnosus* strain V92	98	JF444753
17	Uncultured bacterium isolate DGGE gel band A7	97	GU301189
18	*Lactobacillus similis* gene, strain YIT 12117	94	AB512775

Serial numbers indicate those in the DGGE profiles (Fig. 1A).

Table 4. Identification of yeast and fungi in the *makgeolli* samples by PCR-DGGE.

No.	Nearest neighbor strain	Similarity (%)	Acession No.
1	*Aspergillus tubingensis* strain CNU081066	96	JF411067
2	*Candida glaebosa*	95	FM178351
3	*Saccharomyces cerevisiae* strain ZP 541	100	EU145764
4	*Candida glaebosa*	95	FM178351
5	*Saccharomyces cerevisiae* strain XSQ68-1	100	JF825468
6	*Candida glaebosa*	95	FM178351
7	*Gibberella zeae* isolate Z3639	95	HQ149737
8	*Candida glaebosa*	95	FM178351
9	Uncultured *Candida* clone C4-G8F2-FP	93	HQ646032
10	*Aspergillus niger* isolate South-west 0094	96	FJ537110
11	*Saccharomyces cerevisiae* isolate NN691	100	EU798694

Serial numbers indicate those in the DGGE profiles (Fig. 1B).

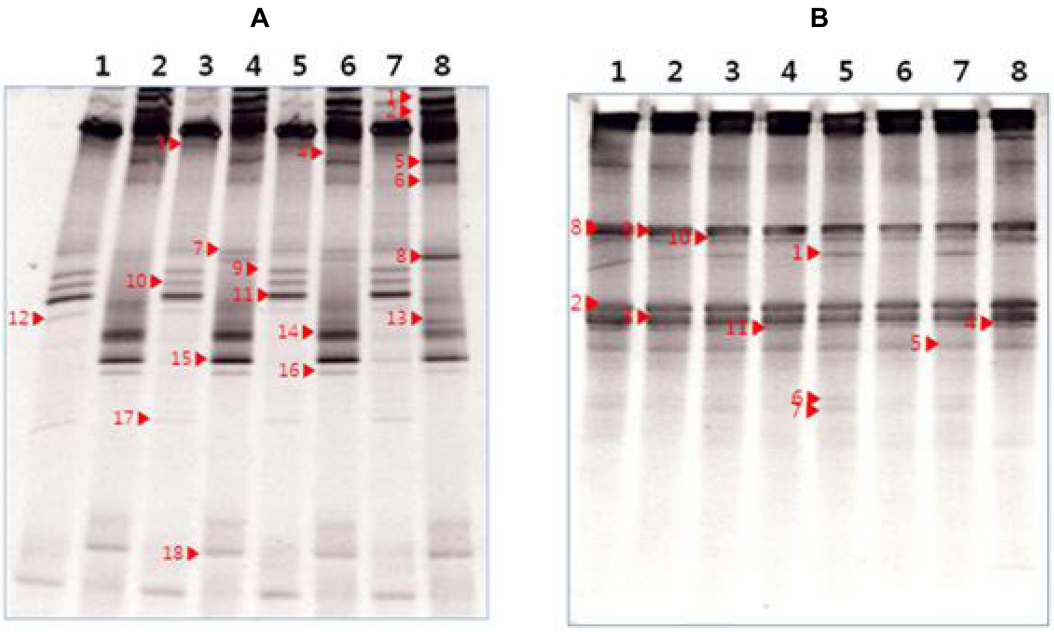

Fig. 1. Profile of DNA bands from *makgeolli* samples by PCR-DGGE (**A**: Bacteria, **B**: Yeast and Fungi).
Lane 1, 4°C, 3 d; Lane 2, 25°C, 3 d; Lane 3, 4°C, 6 d; Lane 4, 25°C, 6 d; Lane 5, 4°C, 9 d; Lane 6, 25°C, 9 d; Lane 7, 4°C, 12 d; Lane 8, 25°C, 12 d. Each number indicates an excised band listed in Tables 3 and 4. Numbers correspond to the nearest neighbor strain designations as presented in the same tables.

(No. 8 band) thickened in proportion to the storage period, and were the darkest bands on day 12. Accordingly, *L. crustorum* and *L. plantarum* were identified as the main microorganisms that affect the deterioration of *makgeolli*. *L. plantarum* can grow abundantly in salted vegetables and dairy products, and the characteristics of its product, bacteriocin, have been widely studied [1, 19]. With its high thermal safety and antibacterial activity within a wide pH range, *L. plantarum* plays a useful role in foods, including *kimchi* and dairy products. However, because *makgeolli* is less preferable when its sour taste increases, *L. plantarum* that exceeds a specific concentration level deteriorates the quality of *makgeolli*. Scheirlinck *et al*. [12] reported *L. crustorum* as a novel *Lactobacillus* species isolated from the traditional Belgian wheat sourdoughs.

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