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Pyrosequencing reveals bacterial diversity in Korean traditional wheat-based *nuruk*§

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The emerging global importance of Korea's alcoholic beverages emphasizes the need for quality enhancement of *nuruk*, a traditional Korean cereal starter that is used extensively in traditional brewing. Apart from fungi and yeasts, bacteria known to be ubiquitously present are also a part of the *nuruk* ecosystem and are known to influence fermentation activity by influencing fermentation favorable factors. In the current study, bacterial diversity and temporal variations in the traditional wheat-based *nuruk*, fermented at two representative temperature conditions for 30 days, along with two commercial wheat-based *nuruk* samples for comparison analysis were evaluated using libraries of PCR amplicons and 454 pyrosequencing targeting of the hypervariable regions V1 to V3 of the 16S rRNA gene. A total of 90,836 16S reads were analyzed and assigned to a total of 314, 321, and 141 Operational Taxonomic Units (OTUs) for *nuruk* A, B, and C, respectively. Diversity parameters clearly indicated *nuruk* B to be more diverse in terms of bacterial composition than *nuruk* A. Taxonomic assignments indicated that *nuruk* A was dominated by phylum *Cyanobacteria*, whereas *nuruk* B was dominated by phylum *Actinobacteria*. For both *nuruk* A and B, members of the phylum *Firmicutes* mostly converged into the family *Bacillaceae*; these microorganisms might be present in negligible numbers at the beginning but became significant as the fermentation progressed. The commercial samples were predominated by phylum *Firmicutes*, which is composed of *Lactobacillaceae* and *Leoconostocaceae*. The findings of this study provide new insights into understanding the changes in bacterial community structure during traditional *nuruk* starter production.

Keywords: wheat, *nuruk*, bacterial diversity, *Firmicutes*, LAB

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Introduction

Traditional Korean alcoholic beverages are mostly brewed using *nuruk*, a Korean cereal starter composed of mixed cultures of naturally incorporated microbes. Among other kinds of cereals, wheat is extensively used in traditional *nuruk* preparation (Yu *et al*., 1998). The increasing worldwide popularity of Korean alcoholic beverages demands both qualitative and quantitative enhancement of the traditional cereal starter. The fermentation of *nuruk* involves the coordinated action of a large diversity of microbes including fungi, yeasts, and bacterial species. During the initial stage of alcoholic fermentation, lactic acid bacteria (LAB) has been known to provide a favorable environment over other bacteria for later stage of alcoholic fermentations, thereby contributing to the characteristic taste and aroma of the beverage (Rhee *et al*., 2011). The acid-forming bacteria in *nuruk* produce an acidic environment, favoring the growth of yeast during the later stage of alcohol fermentation. In addition, the fermentation starter-specific microbial composition in the final alcoholic beverages makes it necessary to study the microbial dynamics of the fermentation starter *nuruk* (Jung *et al*., 2012). Thus, the quality of the final alcoholic beverages is greatly dependent on what the starter has been applied.

Quality enhancement of Korean alcoholic beverages requires proper characterization of the microbial diversity associated with the fermentation starter *nuruk*. This study focuses on the bacterial diversity in traditional wheat-based *nuruk*. Molecular techniques have enabled culture-independent studies of bacterial communities through genomic DNA isolation, followed by amplification and sequencing of hypervariable regions V1 to V3 of the 16S rRNA gene using bacterial-specific primers. The utilization of the 16S rRNA gene as a phylogenetic marker has resulted in clear understanding of vast microbial communities in natural habitats (Costello *et al*., 2009; Roh *et al*., 2010). Numerous studies have illustrated the existence of diverse microflora in different types of *nuruk* (Jin *et al*., 2008; Song *et al*., 2013). Microfloral dynamics in barley *nuruk* during natural fermentation have shown varying amounts of bacteria, yeasts, and molds at different time points (Ponnusamy *et al*., 2013). Since most previous studies were based on classical culture-dependent identification of microflora from different types of *nuruks* and due to the limitations of culturing under standard laboratory conditions, these studies represented only a selective portion of the vast microfloral community present. With the advent of next-generation sequencing (NGS) technology, a thorough investigation of the complete microfloral community in various environmental samples is possible. Recent advances in NGS technology allow for the investigation of

microbial diversity at an unprecedented level of resolution. High-throughput pyrosequencing has been used to analyze the bacterial diversity of several environmental samples (Roh *et al*., 2010; Jung *et al*., 2012; Gołębiewski *et al*., 2014). Although, the bacterial diversity of different types of *nuruks* has been explored, more extensive investigation of the bacterial diversity in traditional wheat-based *nuruk* needs to be done.

Nuruk fermentation is traditionally carried out in the temperature range of 30–45°C (Yang *et al*., 2011; Tamang, 2012). Temperature variation might lead to selective enhancement of particular communities over others. In this study, we applied high-throughput 454 pyrosequencing of the bacterial 16S rRNA gene in two types of traditional wheat-based *nuruk* preparations (i.e., A: 36°C for 30 days and B: 45°C for 10 days, followed by 35°C for 20 days). Thus, this work describes an elaborative pyrosequencing attempt to explore the bacterial diversity associated with traditional wheat *nuruk* under two different fermentation conditions. The results of this study will increase understanding of the composition and variation in the bacteria found in traditional wheat *nuruk* during fermentation.

Materials and Methods

Nuruk preparation and sampling

The traditional Korean wheat cultivar *Keumkang* was used to prepare the initial *nuruk* cake, and two different fermentation conditions were implemented to obtain wheat *nuruk* A and B, as described previously (Bal *et al*., 2014). The same *nuruk* samples were used for metagenomics analysis in this study. Briefly, samples from various time points, including day 0, day 3, day 6, day 10, day 20, and day 30, *nuruk* A and B fermentation were collected and analyzed for bacterial diversity using pyrosequencing of bacterial 16S rRNA gene. Commercially-available wheat *nuruk Sansong*, C01 and *Songhaq*, C02 were also analyzed for comparison purposes.

Metagenomic DNA extraction

Metagenomic DNA was individually extracted from 1 g of each *nuruk* sample. The samples were ground into fine powder and resuspended in 10 ml of lysis buffer (0.05 M Tris-HCl, pH 8.0, 0.05 M EDTA, 1% SDS, and 0.01% 2-mercaptoethanol). The lysates were then incubated at 65°C in a water bath for 10 min with intermittent inverting, followed by centrifugation at 6,500 rpm for 20 min at room temperature (RT). The supernatant was then treated with an equal volume of chloroform/isoamyl alcohol (24:1 v/v) and inverted 20–30 times, followed by centrifugation at 6,000 rpm for 12 min at RT. The supernatant was treated with an equal volume of isopropanol, mixed well, and centrifuged at 6,500 rpm for 12 min at RT. The DNA pellets were then resuspended in 4 ml of TE buffer (10 mM Tris-HCl; pH 8.0 and 1 mM EDTA; pH 8.0) and 40 µl of RNase (10 mg/ml stock solution) and incubated at 37°C for 30 min. To remove further impurities, samples were again treated with an equal volume of PCI, followed by inverting and centrifugation at 6,500 rpm for 12 min at RT. DNA was then precipitated using 2 ml of 7.5 M ammonium acetate and 12 ml of 95% ethanol. The DNA pellets were then washed twice with 70% ethanol, followed by air-drying and resuspension in 100–200 µl of TE buffer. The quantity and quality of isolated DNA were measured using a spectrophotometer and agarose gel electrophoresis, respectively.

Library construction of PCR amplicons and pyrosequencing

PCR amplifications using the primers described in Table 1, which consisted of multiplex identifier (MID) adaptors that facilitated automated sorting of pyrosequencing reads, were carried out in 50 µl reactions, each containing 0.4 pmole of each primer, 25 µl of 2X TOP simple TM premix-HOT (Enzynomics), and 1–5 µl of DNA template (0.35 µg/µl). All PCR reactions were conducted under the following cycling regime: 94°C for 10 min (one cycle); 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min (30 cycles); and 72°C for 10 min (one cycle).

Unwanted short fragments were removed by Agencourt AMPure XP beads (Beckman Coulter) using a 1:1 ratio of DNA and beads. Short-fragment removal was confirmed with the Agilent Bioanalyzer 2100 (Agilent Technologies).

Libraries of PCR fragments were constructed following standard protocols. Quantification was performed using Pico-Green (Life Technologies) with an external Infinite

Table 1. Summary of samples, and details of primer sequences used for pyrosequencing

Sample ID	Barcode sequence	Linker primer sequence	Treatment	Reverse primer
A00	ACGAGTGCGT	ATTACCGCGGCTGCTGG	D00	AGAGTTTGATCCTGGCTCAG
A03	ACGCTCGACA	ATTACCGCGGCTGCTGG	D03	AGAGTTTGATCCTGGCTCAG
A06	AGACGCACTC	ATTACCGCGGCTGCTGG	D06	AGAGTTTGATCCTGGCTCAG
A10	AGCACTGTAG	ATTACCGCGGCTGCTGG	D10	AGAGTTTGATCCTGGCTCAG
A20	ATCAGACACG	ATTACCGCGGCTGCTGG	D20	AGAGTTTGATCCTGGCTCAG
A30	ATATCGCGAG	ATTACCGCGGCTGCTGG	D30	AGAGTTTGATCCTGGCTCAG
B00	CGTGTCTCTA	ATTACCGCGGCTGCTGG	D00	AGAGTTTGATCCTGGCTCAG
B03	CTCGCGTGTC	ATTACCGCGGCTGCTGG	D03	AGAGTTTGATCCTGGCTCAG
B06	TAGTATCAGC	ATTACCGCGGCTGCTGG	D06	AGAGTTTGATCCTGGCTCAG
B10	ACGAGTGCGT	ATTACCGCGGCTGCTGG	D10	AGAGTTTGATCCTGGCTCAG
B20	ACGCTCGACA	ATTACCGCGGCTGCTGG	D20	AGAGTTTGATCCTGGCTCAG
B30	AGACGCACTC	ATTACCGCGGCTGCTGG	D30	AGAGTTTGATCCTGGCTCAG
C01	AGCACTGTAG	ATTACCGCGGCTGCTGG	C01	AGAGTTTGATCCTGGCTCAG
C02	ATCAGACACG	ATTACCGCGGCTGCTGG	C02	AGAGTTTGATCCTGGCTCAG

F200 Pro fluorimeter (Tecan) and the Magellan v7.0 Software (Tecan). Based on the standard concentrations, the signals were directly converted to ng/µl, and the coefficient of determination (validation criteria $R^2 > 0.99$) was calculated from eight DNA standards in a range from 0–100 ng/µl. Equimolar concentrations of each amplicon from different samples were pooled and purified using an AMPure bead kit and then amplified on sequencing beads using emulsion PCR. Recovered beads from emulsion PCR were deposited on a 454 Pico Titer Plate and sequenced with a Roche/454 GS Junior system (SeaSun Biomaterials) following the manufacturer's instructions.

Sequence processing and data analysis

Sequences generated from the pyrosequencing of bacterial 16S rRNA gene amplicons were processed using the Quantitative Insights into Microbial Ecology (QIIME v1.9.0) (Caporaso *et al.*, 2010). Sequencing reads with average quality scores lower than 25, containing one or more ambiguous base calls, or having a length shorter than 200 bp were removed. Operational taxonomic units (OTUs) were assigned using QIIME's USEARCH-based (Edgar, 2010) open-reference OTU-picking workflow, with a threshold of 97% pairwise identity. Taxonomic classification of each OTU up to the genus level was performed by aligning the representative sequences from each OTU with the Greengenes imputed core reference alignment (DeSantis *et al.*, 2006) using PyNAST (Caporaso *et al.*, 2010).

The sequences of OTUs of the 16S rRNA gene of the bacteria in this study were available in GenBank under the accession numbers KU167718-KU167830.

Diversity analysis

Alpha-diversity and beta-diversity estimates were calculated using the QIIME suite of programs. Alpha diversity included *Chao1* richness, Shannon diversity, Simpson diversity, Fisher's alpha, Phylogenetic diversity, Goods coverage, and Observed species (Caporaso *et al.*, 2010). The sequencing depth was also estimated through rarefaction analysis. QIIME was also used to generate weighted UniFrac, unweighted UniFrac, and Bray-Curtis distances matrices. Principal Co-ordinate Analysis (PCoA) plots and boxplots based on these distance matrices were generated using QIIME scripts.

Statistical analysis

The ANOSIM and Adonis tests used to determine the statistical significance related to the diversity analysis were conducted through QIIME scripts.

Results and Discussion

Bacterial diversity during *nuruk* fermentation

454 pyrosequencing of 14 *nuruk* samples, including six each of *nuruk* A and B collected at 0, 3, 6, 10, 20, and 30 days of *nuruk* fermentation and two commercial wheat *nuruk* C01 and C02, yielded a total of 197,383 reads. Following quality trimming, denoising, and chimera removal, 90,836 sequences with average sequence lengths of 412.4 were obtained and subjected to further downstream analysis. The number of valid reads analyzed for each sample varied from 5,295 reads to 8,365 reads (Table 2), which was greater than those (1,181 ± 874 valid reads) of a previously reported pyrosequencing study in *nuruk* (Jung *et al.*, 2012). These sequences were further assigned to a total of 314, 321, and 141 OTUs (as defined by 97% sequence similarity), respectively, for *nuruk* A, B, and C.

Alpha diversity

Alpha diversity indices specifying the richness and/or evenness of various taxa and lineages contained within each *nuruk* sample varied considerably across the three types of *nuruk*. The observed bacterial richness varied from 31 to

Table 2. Alpha diversity indices

Sample	Valid reads	Observed species (S)	Chao1	Shannon's diversity (H')	Shannon's Equitability (E_H)	Simpson's diversity (D_1)	Fisher's_alpha	Goods coverage	PD Whole Tree
A00	5883	31	36	2.1	0.42	0.6	4.37	0.999	1.88
A03	5295	58	66.25	2.87	0.49	0.75	9.11	0.998	2.98
A06	5531	62	71.17	3	0.5	0.77	9.86	0.998	3.1
A10	6559	61	67.11	2.65	0.45	0.7	9.67	0.998	3.01
A20	5656	55	77.75	2.56	0.44	0.68	8.55	0.997	2.84
A30	8365	47	63.5	2.33	0.42	0.65	7.1	0.998	2.54
B00	5653	25	28	2.03	0.44	0.59	3.4	0.999	1.63
B03	6442	58	79	2.98	0.51	0.79	9.11	0.997	2.4
B06	7032	60	61.25	3.12	0.53	0.8	9.48	0.999	2.65
B10	5594	58	62.5	2.7	0.46	0.72	9.11	0.998	2.38
B20	7286	58	59	2.82	0.48	0.77	9.11	0.999	2.39
B30	6795	62	64.33	2.82	0.47	0.78	9.86	0.998	2.54
C01	7983	65	80	2.64	0.44	0.74	10.43	0.997	3.08
C02	6762	76	80.67	4.45	0.71	0.93	12.57	0.998	3.29
Mean	6488.29	55.43	64.04	2.79	0.48	0.73	8.70	1.00	2.62
Mean (A)	6214.83	52.33	63.63	2.59	0.45	0.69	8.11	1.00	2.73
Mean (B)	6467.00	53.50	59.01	2.75	0.48	0.74	8.35	1.00	2.33

62 OTUs in *nuruk* A, 25 to 62 OTUs in *nuruk* B, and 65 to 76 OTUs in *nuruk* C. Rarefaction analysis with an even sequencing depth of 5,295 sequences for all *nuruk* samples indicated adequate sequence coverage to reliably describe the full diversity present in the *nuruk* samples. This result was further confirmed by 100% Good's coverage estimation values. Rarefaction analysis showed a clear-cut asymptotic plateau, indicating adequate sampling and sequencing efforts (Supplementary data Fig. S1). Rarefaction curves showed that *nuruk* B presented greater richness in comparison to *nuruk* A, whereas the richness of the commercial sample C was higher than that of both *nuruk* A and B. Alpha diversity indices specifying the richness, diversity, and evenness of various taxa and lineages contained within each *nuruk* sample were presented in Table 2. The average bacterial richness of *nuruk* A (63.63±14.41), as calculated by *Chao*1, was higher than that of *nuruk* B (59.1±16.77). This result indicated that the *nuruk* A microbiome was higher than the *nuruk* B but it was not statistically significant. Comparison of the average alpha diversity indices such as Shannon's diversity (H'), Simpson diversity (D_1), and Fisher's alpha indicated that the *nuruk* B was more diverse than the *nuruk* A but they were not statistically significant at the level of 5%. Further comparison of evenness through the Shannon's equitability (E_H) index indicated that *nuruk* B was more even than the *nuruk* A but failed to satisfy statistical significance. Since temperature is the major differentiating factor between *nuruk* A and B, the initial fermentation temperature is believed to play an important role in determining the flourishing bacteria in *nuruk* fermentation. Shannon's diversity index values of 3.12 and 3.00, highest among *nuruk* samples B and A, respectively, suggest that the B6 and A6 samples contained the maximum bacterial diversity. In addition to species richness, evenness was also greater in the A6 and B6 samples, as depicted by an E_H of 0.50 and 0.53, respectively. In contrast, the bacterial diversity contained in commercial sample C02 was found to be greater than that of either *nuruk* A or B, as predicted from the H' value of 4.45 and the D_1 value of 0.93. The bacterial branch distance, which measures the complete phylogenetic diversity represented by the index PD_whole_tree within a community, demonstrated significantly higher phylogenetic diversity in *nuruk* C, followed by *nuruk* A and B.

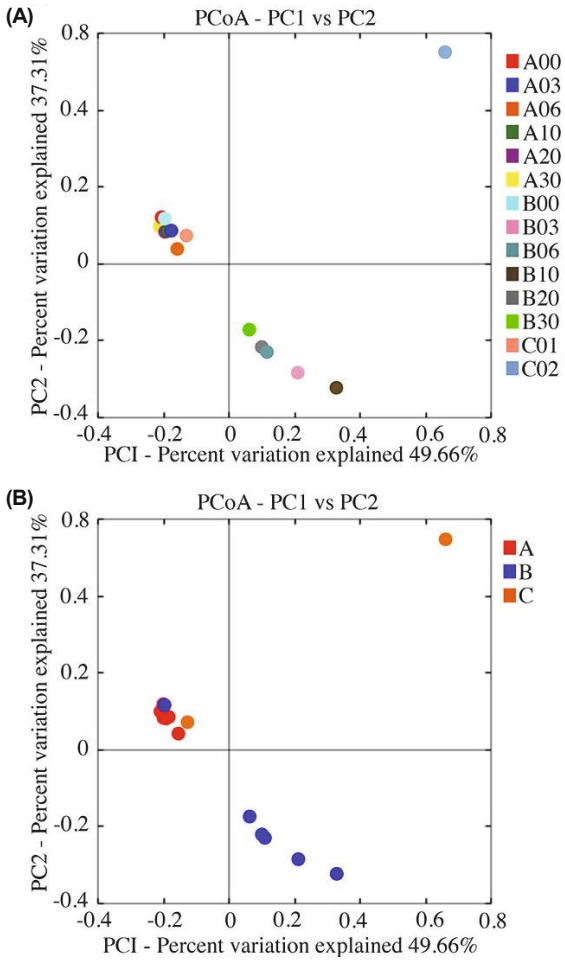

Fig. 1. 2D PCoA plots based on unweighted UniFrac distance matrices. These plots show the putative structural similarities between bacterial communities within (A) different types of *nuruk* at different time points of fermentation and for (B) three different types of *nuruk*.

Beta diversity

The beta diversity patterns were evaluated by plotting two-dimensional PCoA plots based on unweighted UniFrac distance matrices (Fig. 1A and B) generated through QIIME scripts. These plots depicted biome-specific differences among the bacterial communities of *nuruk* A, B, and C. Close to 87% of the variation in the microbial community structures among the different *nuruk* samples was explained by PC1 and PC2. The different bacterial species were clustered together based on the type of *nuruk* (Fig. 1A). The bacterial communities of *nuruk* A and *nuruk* B were shown to be in different clusters, whereas the commercial sample C01 was clustered with *nuruk* A. The commercial sample C02 was

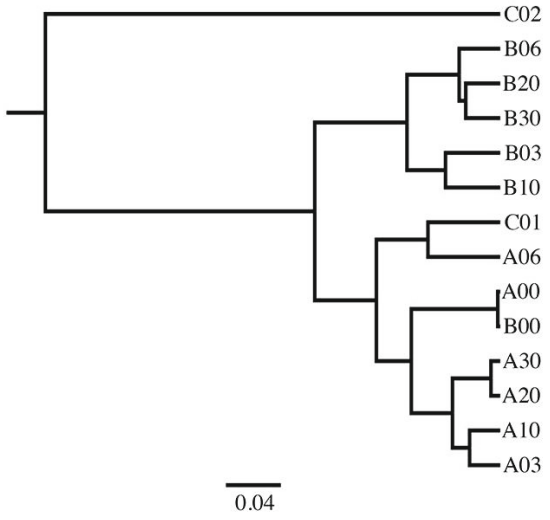

Fig. 2. UPGMA tree plotted from a clustering analysis using UniFrac distance matrices. The tree displays distances between *nuruk* samples at different time points of fermentation. The scale bar indicates the distance between clusters in UniFrac units.

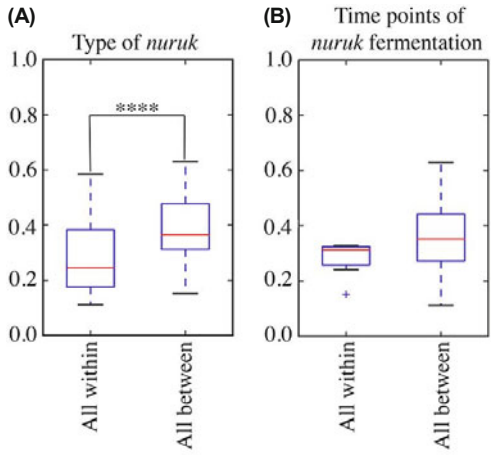

Fig. 3. Boxplots showing unweighted UniFrac distances within and between (A) types of *nuruk* and (B) time points of fermentation. Boxplots span the first and third quartiles, split by the median; whiskers extend to include the farthest outliers. The boxplots present the 25–75 percentile values (box), median (bar inside box), and standard deviation (whiskers)

far from both *nuruk* A and B. The bacterial communities in the day 0 samples were clustered apart from those of the rest of the samples. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree (Fig. 2), which was based on the weighted UniFrac distance matrix and visualized through Figtree (http://tree.bio.ed.ac.uk/software/figtree/), clearly depicted the clustering of samples based on both *nuruk* type and *nuruk* fermentation time point. The bacterial communities in commercial sample C01 were clustered with the day 6 *nuruk* A sample, whereas commercial sample C02 was equidistant from both *nuruk* A and B samples. Boxplots representing distances from unweighted UniFrac distance matrices were constructed using the *make_distance_boxplots.py* script of QIIME in order to observe the differences between and within samples according to the different categories in the mapping file. These plots (Fig. 3A and B) show statistically significant differences (parametric p value <0.0001) within-samples and between-samples for *nuruk* A, B, and C (Fig. 3A). However, no significant difference was observed in plots describing the differences in distances between- and within-samples at the different time points of *nuruk* fermentation (Fig. 3B).

Bacterial community dynamics during *nuruk* fermentation

The taxonomic assignment of each OTU up to the genus level, performed using the QIIME supported Greengenes

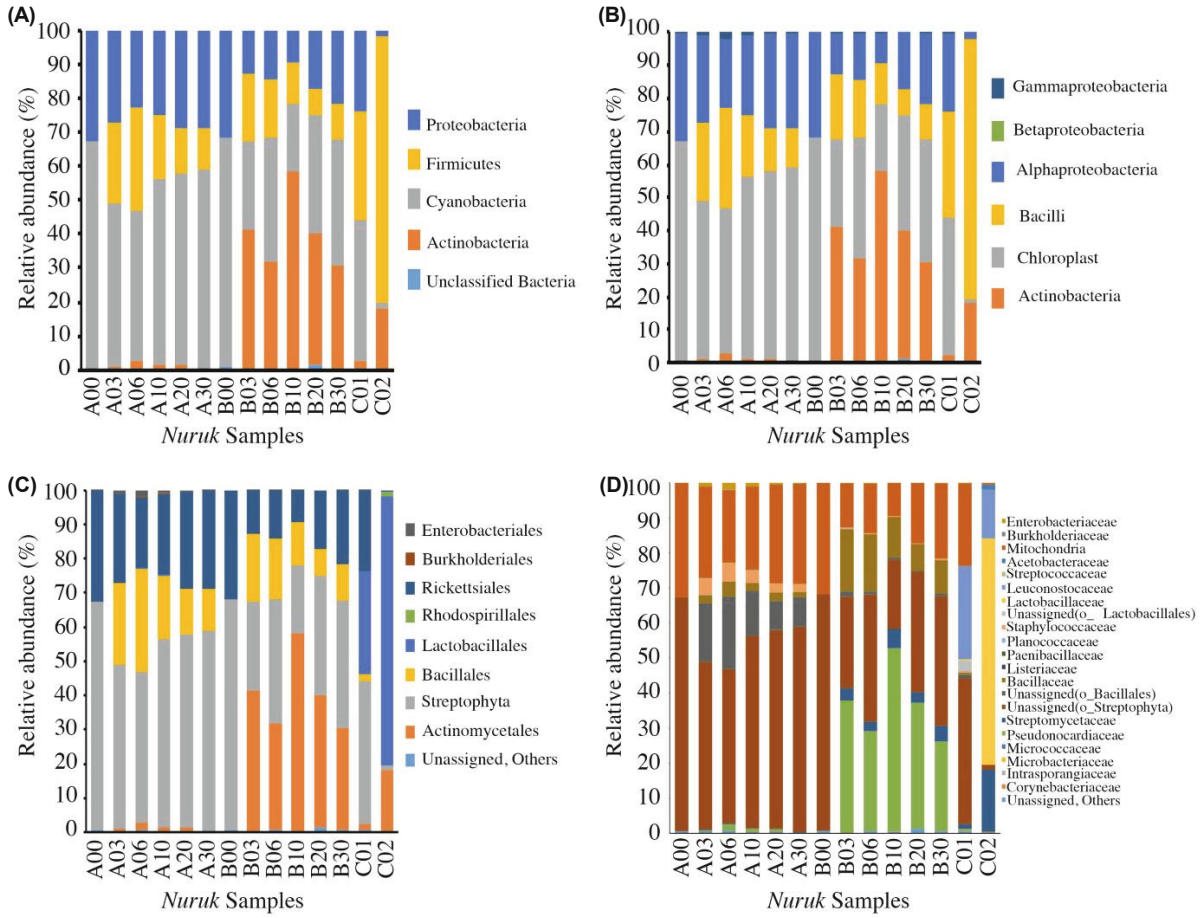

Fig. 4. Bacterial community dynamics based on the taxonomic classification of the 16S pyrosequencing reads at the (A) phylum level, (B) class level, (C) order level, and (D) family level.

(DeSantis *et al*., 2006; MacDonald *et al*., 2012) 16S database, revealed differences in the composition of bacterial microbiomes of different *nuruk* samples. The evaluation of 90,836 valid reads of the 16S rRNA gene following quality trimming, denoising, and chimera removal suggested that the bacteria participating in the *nuruk* fermentation process were classified into the four phyla of *Cyanobacteria*, *Actinobacteria*, *Firmicutes*, and *Proteobacteria* (Fig. 4), a result that is partially consistent with the bacterial diversity previously reported in barley-based *nuruk* (Ponnusamy *et al*., 2013). In contrast to the previous study on the bacterial composition started with six different *nuruks* (Jung *et al*., 2012), *nuruk* A and B differed drastically in the relative abundance of bacteria. Both *nuruk* A and B were predominated by members of the phylum *Cyanobacteria* representing averages of 54.8% (±8) and 36.9% (±16.4), respectively, while the phylum *Firmicutes* was dominant representing an average of 85.6% (±3.2) (Jung *et al*., 2012). Interestingly, the commercial *nuruks* analyzed in this study represented different community dynamics i.e., C01 comprised of three major phyla including *Cyanabacteria* (41.7%), *Firmicutes* (31.9%), and *Proteobacteria* (23.8%), whereas C02 was predominated by *Firmicutes* (78.4%) followed by the second dominant *Actinobacteria* (17.9%). Uncultured *Cyanobacteria* were previously reported as dominant bacteria in the PCR-DGGE profiles of *nuruk* (Kwon and Sohn, 2012). In addition, high incidence of *Cyanobacteria* was observed in sourdoughs, "a mixture of flour and water, spontaneously fermented by LBA and yeasts for bread making" (Ercolini *et al*., 2013). It has been known for the symbiotic colonization of Cyanobacterial isolates with wheat (Gantar *et al*., 1991). Therefore, the large members of the phylum *Cyanobacteria* can be attributed to the fact that the *nuruks* used in this study are predominantly made up of wheat and it will be of an interest to analyze the cereal-specific microbiota. *Nuruk* A was further comprised of the phyla *Proteobacteria* (27.5%), followed by *Firmicutes* (16.4%). Other major phyla dominating *nuruk* B were *Actinobacteria* (33.2%), *Proteobacteria* (17.9%), and *Firmicutes* (11.4%). This observation differed from the previous results (Jung *et al*., 2012) in that *Actinobacteria* and *Proteobacteria* represented only minor percentages and *Firmicutes* was the single dominant phylum. *Actinomycetes* are one of the known cellulase producers (Jang and Chen, 2003; Arunachalam *et al*., 2010). Greater percentages of *Firmicutes* and *Proteobacteria* were observed in *nuruk* A than in *nuruk* B; these percentages also varied as the fermentation progressed. The percentage of *Firmicutes* was negligible in the 0 day samples but significantly increased as fermentation time increased in both *nuruk* A and B. For examples, *Firmicutes* comprised 30% and 20% of the total bacterial population of A06 and B03, respectively. More than 90% of the OTUs belonging to the phylum *Proteobacteria* were of the class *Alpha-proteobacteria*. *Gamma-proteobacteria*, mostly a part of the natural bacterial diversity in soil, plant, and water sources (Ampe *et al*., 1999), constituted around 4.7% of the bacteria in *nuruk* A, whereas it was <1% of the bacteria in *nuruk* B and C. Commercial rice beers were reported to contain less than 1% *Gamma-proteobacteria* (Jung *et al*., 2012). The members of the phylum *Firmicutes* belonged to the class Bacilli in all of the *nuruk* samples but were further segregated into the order *Bacillales* in *nuruk* A and B and *Lactobacillales* in *nuruk* C. At the family level, *nuruk* A was predominated by the unassigned members belonging to the order *Streptophyta* (54.8%) and the *Mitochondria* (26.6%), followed by the unassigned members of the order *Bacillales* and the *Bacillaceae* (Fig. 4). However, *nuruk* B demonstrated different bacterial composition. The family *Pseudonocardiaceae* (30.1%), which was negligible in *nuruk* A, was the second to the unassigned members of the order *Streptophyta* (36.9%). These results were also different from the previous study where *Lactobacillaceae* and *Leuconostocaceae* (53.9% ± 8 and 29.1% ± 10.7, respectively) were two most dominant families in five *nuruk* samples except one (sample ES) with the predominant *Bacillaceae* (78.6%). Interestingly, our commercial *nuruk* C02

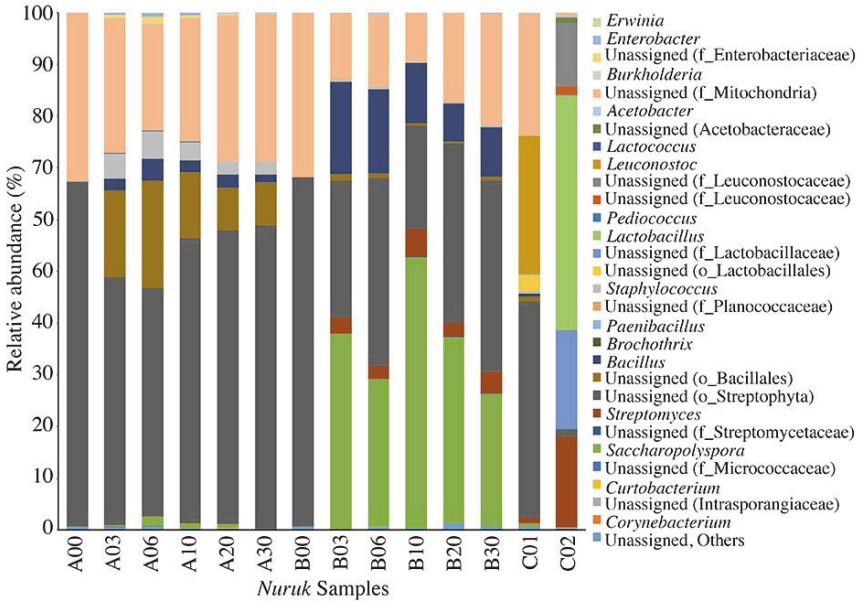

Fig. 5. Bacterial community dynamics based on the taxonomic classification of the 16S pyrosequencing reads at the genus level.

Table 3. Significance tests of the overall effects of sample type and *nuruk* fermentation time on bacterial community structure using two different statistical approaches

Statistical approach	Type of statistics	Sample type	Fermentation time
ANOSIM[α]	R	0.485	0.388
	P	0.001	0.039
Adonis[β]	R^2	0.360	0.742
	P	0.003	0.013

[α] Analysis of similarities ANOSIM.
[β] Non-parametric multivariate analysis of variance with the Adonis function.

revealed the similar composition as the previous study. *Nuruk* C02 was predominated by two families *Lactobacillaceae* (64.5%) and *Leuconostocaceae* (13.8%). These results clearly indicated that, although there were some similarities, fermenting condition resulted in various types of *nuruks* with different microbial communities. The identified bacterial sequences in wheat-based *nuruk* were similar to those reported in the starters of other liquors. *Bacillaceae*, which were dominant in *nuruk* A and B, have been isolated *in vitro* from grapes as alcohol-surviving bacteria (Thomas, 2006) and are also among the group of bacteria found in wine, with its proportion increasing continuously during the fermentation process (Amann *et al*., 1995). In contrast to the bacterial composition of *nuruk* A and B, the LAB family represented by *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae* was found in *nuruk* C and was earlier reported to be present in different types of *nuruk* (Jung *et al*., 2012) and also in other starters such as the one used in the brewing of Fen liquor (Li *et al*., 2013). The prominent genera in *nuruk* A and B were *Bacillus*, *Staphylococcus*, and many unassigned members of the order *Rickettsiales* (Fig. 5). Bacteria belonging to the genera *Bacillus* and *Staphylococcus* were commonly isolated from different types of *nuruk* (Yu *et al*., 1998; Song *et al*., 2013). The genus *Bacillus* is known to assist the saccharification process of rice starch leading to glucose production, which is subsequently utilized by yeast cells in the fermentation process (Lee *et al*., 2005). Unassigned genera of the phylum *Cyanobacteria* were 55% and 37% of *nuruk* A and *nuruk* B populations, respectively. These were also represented around 42% of commercial *nuruk* C02 population. Two genera *Saccharopolyspora* and *Streptomyces* were present with an average percentage of 30% and 3% of the total population of *nuruk* B, respectively. Although *Streptomyces* dominated in *nuruk* C, *Saccharopolyspora* was present in negligible numbers. The commercial sample C01 was predominated by the genera *Leuconostoc* and many unassigned members of the order *Rickettsiales,* whereas C02 was composed of *Lactobacillus*.

Correlations between bacterial communities

Two non-parametric multivariate statistical tests, ANOSIM and Adonis, showed significant variations in bacterial community diversity depending on the type of *nuruk* sample. The bacterial metagenomes in *nuruk* A, B, and C were found to be distinct in composition (ANOSIM R = 0.485 and P = 0.001; Adonis R^2 = 0.360 and P = 0.003; Table 3), a pattern clearly evident from the coordination plot (Fig. 2). These results are consistent with the mycofloral diversity in these *nuruk* samples (unpublished data), implying that temperature during fermentation is a key factor in determining *nuruk* bacterial diversity. In contradiction to the mycofloral diversity in *nuruk* A, B, and C, the bacterial metagenomes in these samples at various fermentation times also varied significantly (ANOSIM R = 0.388 and P = 0.039; Adonis R^2 = 0.742 and P = 0.013).

Conclusion

In conclusion, the bacterial diversity observed in wheat-based traditional *nuruks* undergoing fermentation under two distinct sets of conditions over a period of 30 days was immensely diverse. This study is among the first elaborative 454 pyrosequencing studies of traditional wheat *nuruk*. The detailed bacterial communities formed during the entire starter fermentation process in this study provide us with more definitive information than previous studies that focused only on the finished starter products. Using 454 pyrosequencing, we demonstrated that the fermentation of Korean traditional alcoholic starters is associated with a diverse range of bacteria. The study showed a large shift in bacterial community between the initial samples and samples that underwent fermentation for up to 30 days. The bacterial diversity during the fermentation process clearly showed the selective pressure of the organisms required for the fermentation process. Comprehensively, these results contribute to a broad and clear understanding of the bacterial composition and diversity of Korean traditional wheat *nuruk* and can thereby be applied for the enhancement of *nuruk* fermentation, which will lead to the production of high-quality Korean alcoholic beverages.

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