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Mycoflora Dynamics Analysis of Korean Traditional Wheat-based *Nuruk*

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The growing popularity of traditional Korean alcoholic beverages has led to a demand for quality enhancement of the traditional starter culture *nuruk*, which consists primarily of wheat. Therefore, this study focused on mycoflora characterization and the temporal variations in traditional wheat-based *nuruks* fermented at two representative traditional temperature conditions for 30 days. *Nuruk* A was fermented at a constant temperature of 36°C for 30 days and *nuruk* B was fermented at a high initial temperature of 45°C for 10 days followed by 35°C for 20 days. The average mycoflora load in the two different *nuruk* conditions did not vary significantly between the 0 and 30 day cultures, and a maximum load of 8.39 log CFU/g was observed for *nuruk* A on culture day 3 and 7.87 log CFU/g for *nuruk* B on culture day 30. Within two samples, pH was negatively correlated with temporal changes in mycoflora load. The pH of *nuruk* A was significantly lower than that of *nuruk* B at all of the time points evaluated. Culture-dependent characterization led to the identification of 55 fungal isolates belonging to 9 genera and 15 species, with the most prominent genera comprising *Lichtheimia*, *Penicillium*, *Trametes*, *Aspergillus*, *Rhizomucor*, and *Mucor*. A total of 25 yeast isolates were characterized belonging to 6 genera and 7 species, the most prominent among which were *Rhodotorula*, *Pichia*, *Debaryomyces*, *Saccharomycopsis*, and *Torulospora*. Mycofloral community dynamics analysis revealed that both samples A and B varied considerably with respect to the fungal communities over a span of 30 days.

***Keywords*:** wheat, *nuruk*, mycoflora, *Lichtheimia*, *Aspergillus*

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Introduction

Nuruk is a starter culture made with wheat flour and is fermented spontaneously by mixed inocula of environmentally-incorporated microbes. *Nuruk* is used extensively for brewing the cereal-based Korean alcoholic beverages *Makgeolli* and *Takju*. The increasing global popularity of *Makgeolli* in recent years has focused attention on enhancing the quality of traditional *nuruks*, which can be accomplished through proper characterization and community dynamics of *nuruk* microflora present in the fermentation process. Traditional *nuruk* is prepared in the form of cakes from unsterile dough of coarsely ground grains primarily wheat (Yu *et al.*, 1998; Lee *et al.*, 2009). A diverse array of microorganisms including bacteria, fungi and yeasts are naturally associated with *nuruk* during the fermentation process. *Makgeolli* fermentation using *nuruk* as a starter is a two-step process that includes saccharification by fungal species and alcoholic fermentation by yeasts (Yu *et al.*, 1998). During this fermentation, the fungal community acts as the major source of amylolytic and proteolytic enzymes for the saccharification process (Park *et al.*, 1995) while yeasts facilitate alcoholic fermentation. In addition, fungi and yeasts play an important role in conferring flavor and aroma to alcoholic beverages (Feron *et al.*, 1996; Li *et al.*, 2013).

Several studies have been conducted to analyze the *nuruk* microflora (Yu *et al.*, 1998; Yang *et al.*, 2011; Ponnusamy *et al.*, 2013; Song *et al.*, 2013). Microflora characterization in a variety of *nuruks* collected from various provinces in Korea has demonstrated a diverse population of bacteria, fungi, and yeast (Song *et al.*, 2013). While the microbial community dynamics in barley *nuruk* during natural fermentation have been shown to be composed of varying amounts of bacteria, yeasts and molds at different time points (Ponnusamy *et al.*, 2013), the mycoflora dynamics of traditional wheat *nuruk* have not been analyzed previously.

Nuruk fermentation is primarily carried out at a temperature range of 30–45°C (Yang *et al.*, 2011; Tamang, 2012). Temperature variation may lead to selective enhancement of particular communities over others. In this study, we evaluated two different methods of traditional wheat *nuruk* preparation employing different temperatures and characterized the mycoflora dynamics associated with the two methods throughout a 30 day fermentation process. The results of this study should help us to better understand the distribution of mycoflora in traditional wheat *nuruk* during fermentation and thus help to identify important factors for quality enhancement of traditional alcoholic beverages.

Materials and Methods

Nuruk preparation and collection

Traditional Korean wheat cultivar *Keumkang* was used and preparation of the initial *nuruk* cake was performed in a traditional manner as described previously (Buglass, 2011). Two different fermentation conditions were implemented to obtain traditional wheat *nuruk*. Briefly, *nuruk* cakes were incubated at 36°C for a period of 30 days with intermittent inverting at an interval of 2 days (*nuruk* A). As a comparison, other *nuruk* cakes were incubated at 45°C for 10 days and then 35°C for 20 days with intermittent inverting at an interval of 2 days (*nuruk* B). Samples from different time points including day 0, 3, 6, 10, 20, and 30 of *nuruk* A and B samples were collected for mycoflora analysis. Commercially-available wheat *nuruk Sansong*, C1 and *Songhaq*, C2 were also analyzed for comparison purposes.

pH estimation of *nuruk* samples

Changes in physicochemical parameters such as pH were estimated in all *nuruk* samples by homogenizing 1 g of *nuruk* sample in 10 ml normal saline solution (NSS) and incubating at 4°C for 8 h followed by centrifugation. The pH of the resulting supernatant was measured using a pH meter (Beckman Coulter, USA).

Enumeration of mycoflora

Five grams of a cube extracted from the center of the *nuruk* cake, which included portions from the upper and lower surfaces as well as the central core, were suspended in NSS and homogenized manually for 1 h followed by decanting the suspension into sterile tubes. The suspensions were then serially diluted and each diluent was plated in triplicate on different media such as Dichloran Rose-Bengal Chloramphenicol Agar (DRBC, BD Biosciences, USA), Dichloran-Glycerol 18% (DG18, MB Cell, Korea), Potato Dextrose Agar (PDA, BD Biosciences), PDA+Deoxycholate, and YPD agar. Plates were incubated for 3–4 days at 25°C, after which fungal and yeast colonies were enumerated.

Mycoflora characterization

A combination of morphological and molecular analysis was performed to characterize the mycoflora present in the *nuruk* samples. To identify fungal isolates, each single morphologically different isolate was selected and inoculated into a new PDA plate and cultured at 25°C for 7 days. Morphological analysis included colony morphology, differences in pigmentation, and mycelia characteristics. For molecular identification, sequence analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal gene (rDNA) was conducted as described previously (White *et al.*, 1990; Song *et al.*, 2013). For filamentous fungi, mycelia were harvested and freeze dried followed by genomic DNA extraction. To identify yeast isolates, isolated colonies were streaked onto new YPD plates and incubated at 30°C for 3 days followed by genomic DNA extraction. PCR was carried out to clone the ITS1-5.8S-ITS2 rDNA region using the following primer pair; forward ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and reverse ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). For yeast isolates, the D1/D2 domain of 26S rDNA region was amplified using the following primer pair; NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (White *et al.*, 1990). PCR products were electrophoresed followed by elution of the DNA band of appropriate size. The eluted DNA was then sequenced using ITS1 and ITS4 primers for fungal isolates and NL1 and NL4 primers for yeast isolates. Sequences were compared using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

Traditional wheat *nuruk* fermentation conditions and pH variations

Nuruk fermentation is typically carried out at a continuous temperature range of 30–45°C (Yang *et al.*, 2011; Tamang, 2012). It is well known that temperature variation leads to selective enhancement of particular communities over others, and also that high initial temperatures help to prevent contamination (Im *et al.*, 2014). Therefore, we employed two different traditional wheat *nuruk* preparations using different temperatures. *Nuruk* A was fermented at a constant temperature of 36°C for 30 days and represented the traditional continuous fermentation condition. For comparison, *nuruk* B was fermented at a high initial temperature of 45°C for 10 days followed by 35°C for 20 days. The high initial temperature of *nuruk* B was applied to prevent unwanted competing microbes that outnumber the favorable fermentative mycoflora at 35°C. Both *nuruk* A and *nuruk* B were evaluated to characterize the mycoflora dynamics of the fermentation process for 30 days.

pH is an important physico-chemical parameter influencing microbial growth (Rousk *et al.*, 2009) and is of special concern in the case of fermented food products since changes in pH impart a selective effect on the growth of microbial communities (Coton and Leguerinel, 2014). *Aspergillus flavus* isolates produce more aflatoxins at acidic pH and so is the case with

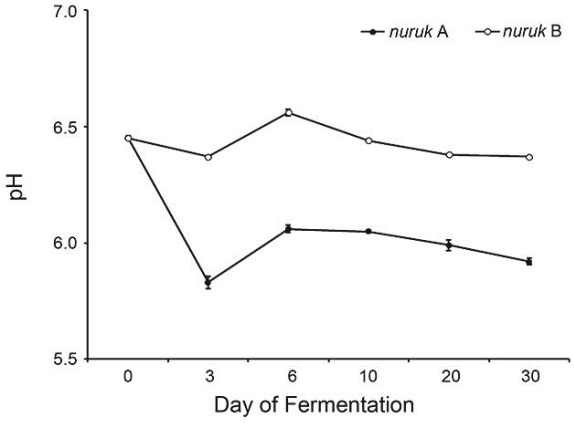

Fig. 1. Interdependence of pH on mycofloral diversity. Changes in pH estimated in all *nuruk* samples. *nuruk* A and *nuruk* B after 0, 3, 6, 10, 20, and 30 days.

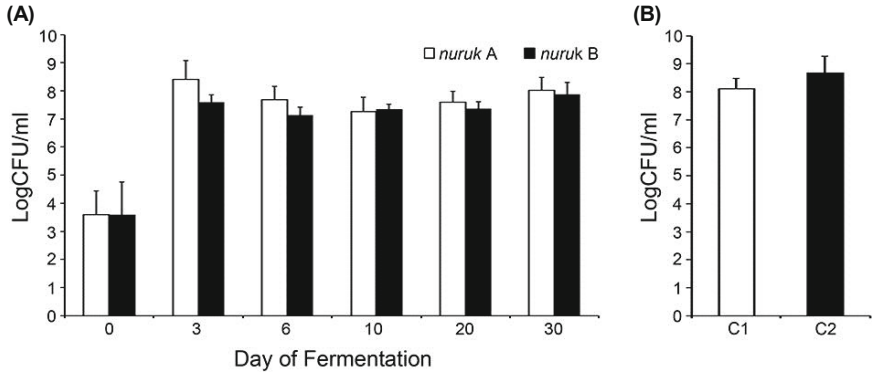

Fig. 2. Mycoflora dynamics of cultured filamentous fungi in traditional wheat *nuruk*. (A) Enumeration of mycoflora in *nuruks* prepared under two different conditions: *nuruk* A was incubated at 36°C for a period of 30 days, while *nuruk* B was incubated at 45°C for 10 days and then at 35°C for 20 days. (B) Mycofloral load in commercially-available wheat *nuruk* samples.

Penicillium roqueforti. Thus, variations in pH were measured for both cultures at 0, 3, 6, 10, 20, and 30 days. Both cultures exhibited a decrease in pH after 3 days of culture followed by a slight increase on the 6th day (Fig. 1). The temporal variations in pH correlated with that of mycoflora load. Specifically, increased mycoflora load was associated with decreased pH and *vice versa*. In addition, the pH of the *nuruk* B culture was consistently and significantly higher than that of *nuruk* A throughout the 30 day fermentation period. As a reference, the pH values of the commercial *nuruks* C1 and C2 were 6.29 and 6.61, respectively.

Characterization of mycofloral dynamics during traditional wheat *nuruk* fermentation

For proper representation of mycoflora, we used 5 g of a central cube cut from the *nuruk* cake, because it included portions from both the upper and lower surfaces as well as the central core. Apart from DRBC agar medium, other media such as YPD, PDA, DG18, and PDA+D were not free from bacterial growth, which interfered with the growth of fungi and yeasts (data not shown). DRBC proved to be the best medium to support controlled growth of fungi and yeasts without any kind of bacterial contamination (King *et al.*, 1979). The total mycofloral load as estimated from the growth in DRBC was not significantly different in the two different *nuruks* (Fig. 2) between fermentation day 0 and 30. There was an initial increase in mycofloral load from 3.59 log CFU/g to 8.39 log CFU/g in the case of *nuruk* A and 7.59 log CFU/g in the case of *nuruk* B on day 3, followed by a decrease to 7.25 log CFU/g and 7.34 log CFU/g, respectively, on day 10, becoming almost stationary thereafter up to day 30. In general, *nuruk* B was slightly less populated than *nuruk* A. In comparison, the mycofloral loads of the commercially available wheat *nuruks Sansong* C1 and *Songhaq* C2 were slightly higher than ours at 8.11 and 8.68 log CFU/g, respectively.

Following enumeration, identification and characterization of the mycoflora associated with the *nuruk* samples was performed by morphological and molecular analysis. Based upon differences in colony morphology, mycelial pattern, and pigmentation, 87 different filamentous fungi were isolated from our *nuruk* samples and transferred into fresh PDA plates. After growing the pure culture on PDA, the 87 isolates were divided into 33 groups depending upon similarities in colony morphology. At least two representative isolates for each group (except for those with a single isolate) were classified based on sequence identity of the ITS region of the rDNA in the GenBank database (Table 1). A total of 55 isolates were identified as belonging to 9 genera and 15 species (Table 1). Among them the predominant genera *Lichtheimia* comprised 11 isolates, while *Aspergillus* comprised 28 isolates, and *Trametes* and *Rhizomucor* comprised 5 and 4 isolates,

Table 1. Predominant fungal and yeast isolates from traditional wheat *nuruk*

Fungi isolated from nuruk samples	No. of isolates	Yeast isolated from nuruk samples	No. of isolates
Lichtheimia corymbifera	8	*Rhodotorula mucilaginosa*	1
Lichtheimia ramosa	3	*Pichia anomala*	3
Mycocladus corymbiferus	1	*Debaryomyces hansenii*	14
Penicillium roqueforti	2	*Wickerhamomyces anomalus*	1
Trametes versicolor	4	*Saccharomycopsis fibuligera*	4
Irpex lacteus	1	*Pichia burtonii*	1
Fusarium oxysporum	1	*Torulaspora delbrueckii*	1
Penicillium viridicatum	1		
Aspergillus flavus	3		
Aspergillus niger	1		
Aspergillus ruber	1		
Trametes sp.	1		
Rhizomucor pusillus	4		
Aspergillus oryzae	4		
Aspergillus tritici	19		
Mucor circinelloides	1		

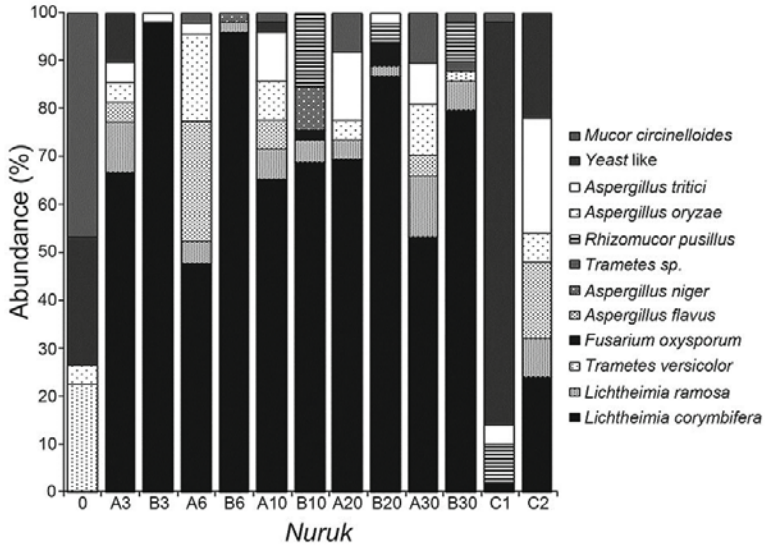

Fig. 3. Mycofloral community dynamics of *nuruk* A, *nuruk* B, and commercially-available *nuruk* C1 and C2. Comparison of community dynamics of *nuruk* A and B samples was conducted. Samples at 0, 3, 6, 10, 20, and 30 days of fermentation were collected from *nuruk* A and indicated as 0, A3, A6, A10, A20, and A30, respectively. The corresponding *nuruk* B samples were also indicated as B3, B6, B10, B20, and B30, respectively. Commercially-available *nuruk Sansong*, C1 and *Songhaq*, C2 were also included for comparison.

respectively. Consistent with these results, several genera such *Lichtheimia* and *Aspergillus* have been reported as being predominant fungi associated with *nuruk* (Uchimura *et al.*, 1990; Park *et al.*, 1995; Yang *et al.*, 2011)

Apart from the filamentous fungal isolates, the D1/D2 domain of 26S rDNA region was amplified using primers NL1 and NL4 in order to characterize the yeasts present in the *nuruk* samples. A total of 25 different isolates were selected on the basis of single cell morphology under microscopic examination, which were further characterized using sequence identity of the D1/D2 domain of 26S rDNA region in the GenBank database to identify 6 genera and 7 species (Table 1). The most noticeable among the yeasts were *Debaryomyces hansenii* with 14 isolates followed by *Saccharomycopsis fibuligera* with 4 isolates and *Pichia anomala* with 3 isolates. Consistent with these results, the Korean starter *nuruk* has previously been reported to contain a number of the same yeasts including *D. hansenii*, *P. anomala*, and *S. fibuligera* (Boekhout and Robert, 2003).

P. anomala isolated from traditional *nuruk* has been reported to produce higher levels of alcohol than other species (Ho *et al*., 2013). According to our sequence comparison in the GenBank database, one of the isolates was identified as *Wickerhamomyces anomalus*, which was recently renamed from *P. anomala* (Kurtzman, 2011). *W. anomalus* has also been reported to possess an active beta-glucosidase that plays a role in wine fermentations (Quatrini *et al.*, 2008; Swangkeaw *et al.*, 2009). In addition, one of the isolates included the non-Saccharomyces yeast *Torulaspora,* which is an industrially important yeast used in the wine and bread making industries (Tataridis *et al.*, 2013). In addition to *Torulaspora*, we also isolated *Rhodotorula mucilaginosa,* which is currently being evaluated as a potential candidate for bioconversion of lignocellulosic sugars to biochemical (Vajzovic *et al.*, 2012; Díaz *et al.*, 2013).

Mycofloral community dynamics analysis was performed in order to obtain a clear view of the temporal distribution of the fungi and yeasts in both types of *nuruk* over a span of 30 days (Fig. 3). A random sample of 50 colonies was isolated and characterized based upon their colony morphology, mycelial growth pattern, and pigmentation in comparison to that of already identified isolates from each *nuruk* sample, followed by determination of their relative abundance. Both samples A and B varied considerably with respect to the fungal communities present, even though *Lichthemia* was the predominant genus. Indeed, *Lichthemia* was among the predominantly and consistently isolated genera from *nuruk* (Uchimura *et al.*, 1990; Park *et al.*, 1995; Yu *et al.*, 1998). In the present study, *Lichthemia corymbifera* comprised 48% and 68% of the *nuruk* A and *nuruk* B mycofloral loads, respectively. This result was in accordance with the fact that *Lichthemia* prefers high temperatures for growth and that *nuruk* B was initially fermented at 45°C for 10 days (Fig. 3). Although *Lichtheimia* was not detected at day 0, its presence was strongly observed throughout the 30 days of fermentation. We suspect that the initial absence of *Lichtheimia* may have been due to the limitations of our culture-dependent identification method.

In addition to *Lichtheimia*, the *Aspergillus* genera constituted a substantial presence at all time-points in *nuruk* A compared to *nuruk B,* among which *A. oryzae* and *A. tritici* were prominent. On the other hand, *R. pusillus* was abundantly present in *nuruk* B. The mycofloral community in *nuruk* A consisted of both fungi and yeasts was more diverse than that of *nuruk* B, in which yeasts were scarcely present. *A. flavus*, which is known to produce aflatoxin, comprised approximately 6% of the mycofloral load in *nuruk* A, whereas it was absent in *nuruk* B. It is quite interesting and surprising that *A. flavus* was found in one of the *nuruk* samples. In addition to ITS sequence analysis, further studies using multiple sequence analysis need to confirm the molecular taxonomy. Since all strains of *A. flavus* are not aflatoxigenic, further studies using a multiplex RT-PCR approach to detect aflatoxigenic strains of *A. flavus* (Degola *et al.*, 2007) followed by aflatoxin estimation assay through biochemical analysis of our *nuruk* samples will clarify the quality control aspects of our *nuruk* samples. More astonishingly, the microbial community dynamics of the commercial samples were completely different from that of *nuruk* A and B samples. Specifically, both C1 and C2 consisted predominantly of yeast-like species (Fig. 3).

In conclusion, this study described the mycofloral diversity of traditional wheat based *nuruk* fermented at two different temperatures over a span of 30 days and provided a comparison with commercial samples. To augment several existing mycoflora characterizations of wheat based traditional *nuruk*, this study presented a detailed mycoflora analysis of the temporal variations that occur throughout a 30 day *nuruk* fermentation period. Our results suggest that both *nuruk* A and B are rich in a diverse array of fungi and yeasts, many of which have been previously reported to be associated with other types of *nuruk*. Although this study characterized several fungal and yeast genera associated with *nuruk*, this experiment also described the variation of mycoflora upon modulation of fermentation conditions of *nuruk,* which may provide for a better understanding of how to improve culture conditions in order to enhance the quality of Korean alcoholic beverages. While this was a detailed study, due to limitations of culture dependent identification, many more genera could have been isolated. In the future, metagenomic approaches will be necessary to substantially clarify the microbial population, gene content, metabolic potential, and functions of microbial communities in traditional *nuruk*.

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