The microbial community and the metabolites of barley nuruk were studied to determine the time-dependent correlation between the fermentation of microbes and metabolites. Samples were analyzed by a polyphasic approach based on culture-dependent, culture-independent (PCR-DGGE and qPCR analysis), and metabolite analysis using GC-MS. Barley nuruk consists of varying amounts of bacteria, yeasts, and molds. The PCR-DGGE results showed that only one phytype, Aspergillus oryzae, was predominant throughout fermentation, reaching a maximum on day 9. The bacterial load was higher on day 6 of fermentation, and then gradually decreased because of increased fungal activity. The shift in fungal and bacterial diversity observed by DGGE was further confirmed by qPCR analysis. In addition, microbes closely related to Pantoaea agglomerans and Saccharomyces fibuligera appeared to play key roles in the fermentation of barley nuruk. GC-MS analysis combined with multivariate analysis, including PCA, PLS-DA, and OPLS-DA, showed fermentation time-dependent metabolite patterns. A total of 21 metabolites, including organic acids, amino acids, sugars, and sugar alcohols, were identified. In particular, glycerol, malic acid, fructose, glucose, sucrose, and maltose were produced at the early fermentation stages (0–6 d), whereas glutamine, aspartic acid, glutamic acid, mannitol, and xylitol were produced during the latter stages of fermentation (9–18 d). Mixed culture fermentation was found throughout the natural fermentation of barley nuruk starter. Most likely, A. oryzae had a major role in saccharification, along with other mixed cultures.

Key words: barley nuruk starter; microbial community; DGGE; metabolites; GC-MS

The preliminary breakdown of starch into simple sugars by single culture fermentation (mostly Aspergillus oryzae) is called koji fermentation. On the other hand, mixed cultures by means of natural fermentation are referred as nuruk starter fermentation, a first step in most of the cereal-based traditional Korean alcoholic beverages, makgeolli and takju. In general makgeolli is prepared using rice as raw material; wheat and barley are also used as alternative cereals. Nuruk fermentation consists of a mixed culture of microorganisms, including fungi, yeast, and bacterial species. A small amount of earlier fermented solid material is added as inoculum to steamed rice, barley, soybean or wheat for saccharification. Microbial fermentation reduces the level of antinutrients such as polyphenols and phytic acids. Various fermented foods (miso, tofu and tempeh) are known to have improved digestibility of carbohydrates, protein, enhanced bioavailability of simple sugars, minerals, and secondary metabolites. Fermented foods have additional value such as antitumor, antibacterial (ferulic acid), and antioxidant properties (terphenyllin). They are either derived from raw materials or the secondary metabolites of the fermenting microorganisms.

The microbial dynamics of starter barley nuruk during natural fermentation must be understood to select the desirable time point for the next step of alcohol fermentation. Hence, the microbial community and the quantity have to be monitored throughout fermentation to get desirable products, and at the same time to avoid undesirable microbes. The classical culture-dependent method is used widely for microbial community analysis, but this single method has limitations and the polyphasic approach minimize, the biases of microbial diversity from mixed culture. High-throughput molecular biology techniques such as PCR based community DNA analysis with denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qPCR) provides more insight into microbial diversity and shifts in the fermentation medium.

Microbiological and enzymatic processes contribute to the production of desirable metabolites that generate unique aromas, flavors, and textures in the natural fermentation of complex foods. Metabolomics studies offer quantitative profiling of metabolites in a complex biological system, but studies of the metabolite profile of barley nuruk starter are scarce. Few studies of rice nuruk starters or other beverages have focused on microbial or metabolite profiles. The combined study of microbial dynamics and its influence on the saccharification of food and metabolite development is needed for the recommendation or selection of effective starters. In the present study, a detailed comparative investigation of barley nuruk starter fermentation was carried out based on culture-dependent and culture-independent methods of microbial divergence and metabolic analysis by GC-MS.

Materials and Methods

Sample collection. Traditional barley nuruk starters were collected at different fermentation times during June 2010. A grain sampler was used to collect samples from various locations and depths in fermentation boxes. A pH meter (Thermo Fisher Scientific, Fremont, CA) was used to measure the pH of the barley nuruk samples.

1 To whom correspondence should be addressed. Tel: +82-2-2049-6177; Fax: +82-2-455-4291; E-mail: chlee123@konkuk.ac.kr
Table 1. PCR Primers Used to Amplify Bacterial and Fungal Genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’ → 3’)</th>
<th>Amplicon size (bp)</th>
<th>Annealing temp. (°C)</th>
<th>Target group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>968F</td>
<td>*AACGGCGAACACCTTAC</td>
<td>433</td>
<td>56</td>
<td>Bacteria 16S DNA (V3–V8)</td>
<td>11)</td>
</tr>
<tr>
<td>1401R</td>
<td>GCGTGTTGACGCAAAGCC</td>
<td>1200</td>
<td>56</td>
<td>Bacteria 16S DNA (V1–V3)</td>
<td>12)</td>
</tr>
<tr>
<td>341F</td>
<td>CCTTGGGGGACGGCACGCG</td>
<td>585</td>
<td>52.5</td>
<td>Eucarya (26S of mold and yeast)</td>
<td>13)</td>
</tr>
<tr>
<td>907R</td>
<td>TCAATCCCTTGTGTATTT</td>
<td>467</td>
<td>60</td>
<td>Bacteria 16S DNA</td>
<td>14)</td>
</tr>
<tr>
<td>Euk 63F</td>
<td>*GCCATATCAATAAAGGGGAAAG</td>
<td>223</td>
<td></td>
<td>Aspergillus oryzae calmodulin gene specific</td>
<td>this study</td>
</tr>
<tr>
<td>Euk 266R</td>
<td>ATTTCCCAACAACCTGAC</td>
<td>561</td>
<td></td>
<td>Aspergillus oryzae KCCM 60345</td>
<td>2639)</td>
</tr>
<tr>
<td>338F</td>
<td>ACTTCCCTGAGGGGACG</td>
<td>467</td>
<td>60</td>
<td>Aspergillus oryzae KCCM 60345</td>
<td>2639)</td>
</tr>
<tr>
<td>805R</td>
<td>GACTACAACGTATCTTAACCC</td>
<td>109</td>
<td>61</td>
<td>Aspergillus oryzae KCCM 60345</td>
<td>2639)</td>
</tr>
<tr>
<td>AocF</td>
<td>ATATCGAGCAGCAGATACACCCAAA</td>
<td>566</td>
<td></td>
<td>Aspergillus oryzae KCCM 60345</td>
<td>2639)</td>
</tr>
<tr>
<td>AocR</td>
<td>TGTGCGCGTCAACCTGTTAATAC</td>
<td>561</td>
<td></td>
<td>Aspergillus oryzae KCCM 60345</td>
<td>2639)</td>
</tr>
</tbody>
</table>

The following type strains were used as positive controls: 1) Enterococcus faecalis (KACC 13807) and 2) Aspergillus oryzae KCCM 60345.

Enumeration and isolation of culturable microorganisms. Samples (0.5 g) were mixed with 4.5 mL of sterile saline solution, soaked at 4 °C for 30 min, and mixed. Serially diluted samples were plated on Luria Bertani (LB) agar medium supplemented with cyclohexamide (0.001%; Sigma-Aldrich, Seoul, Korea) to isolate bacteria. Yeast Mannitol (YM) agar (Difco Laboratories, Detroit, MI) medium was used to isolate fungi, and chlorotetracycline (0.01%; Sigma-Aldrich, Seoul, Korea) and chloramphenicol (0.001%; Sigma-Aldrich, Seoul, Korea) were added to plates to prevent bacterial growth. In order to identify bacterial species, cultures were purified and subjected to molecular identification using universal bacterial and fungal primers 16S and 26S rDNA respectively (Table 1).

Standardization of DNA extraction from barley nuruk samples. The barley nuruk samples were washed in phosphate buffer for 20 min and centrifuged at 4,000 rpm for 10 min, and the supernatant was collected. This was repeated 5 times, and the collected supernatant was centrifuged at 15,000 rpm for 15 min. The pelleted cells were digested with lyticase enzyme (Sigma-Aldrich, Seoul, Korea) and incubated at 37 °C for 1 h, followed by SDS (2%, w/v) digestion for 45 min at 65 °C. The mixture was mechanically disrupted with zirconium beads for 20 s. This process was repeated 5 times. After centrifugation, an equal volume of the following solutions was added to the supernatant: chloroform:isoamyl alcohol (24:1) and phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated with isopropanol and ethanol (25:24:1). DNA was precipitated with isopropanol and ethanol (25:24:1). DNA was precipitated with isopropanol and ethanol (25:24:1). DNA was precipitated with isopropanol and ethanol (25:24:1).

PCR amplification of bacteria and fungi. The 16S rRNA gene was amplified using GC-clamped universal primers 968F and 1401R, as outlined in Table 1. The PCR reaction buffer consisted of universal primers (0.18 μM), dNTPs (0.25 mM), Es Taq polymerase (0.75 U; Takara, Ohtsu, Japan), MgCl2 (2 mM), and DNA template (10 ng). Eukaryote-specific (63f-2666) GC-clamp primers were used to amplify mold and yeasts. The primer specific PCR annealing temperatures are listed in Table 1. The amplicon (5 μL) was analyzed by agarose gel electrophoresis (1.2%, w/v) prior to DGGE analysis.

Denaturing gradient gel electrophoresis. PCR samples were loaded onto 8% polyacrylamide gels in 1× TAE at a 45–55% urea and formamide denaturing gradient (7 urea and 40% (V/V) formamide, corresponding to a 100% gradient) as described elsewhere. 1-2 Electro- phoresis was performed at 60 °C at a constant voltage.1-3 The gels were stained with SYBR Green I (Invitrogen, Eugene, OR), observed under UV transillumination, and documented with a Molecular Imager Gel Doc (BioRad Laboratories, Hercules, CA). The PCR amplicons of the type strains and a representative barley sample were included as markers to serve as standards. The following type strains possessing different G + C%, were obtained from the Korean Agricultural Culture Collection (KACC) or Korean Collection for Type Cultures (KCTC): Bacillus fragilis (KCTC 3688), Bacillus vulgatus (KCTC 24077), and Enterococcus faecalis (KACC 13807), Escherichia coli (KCTC 1682), Lactobacillus acidophilus (KACC 12419), Lactobacillus casei (KACC 12413), Candida albicans KACC 30062, Saccharomyces cerevisiae KCTC 790C, Aspergillus oryzae KCCM 60345, Aspergillus niger KACC 40280, and Aspergillus sojae KACC 40072. Unique, repeated bands were PCR-amplified without a GC clamp and purified before sequencing (Macrogen, Seoul, Korea).

Phylogenetic analysis. Sequences were analyzed using a Chroma- sPro (Technelysium, Australia) and Sequence Scanner V1.0 (Applied Biosystems, Foster City, CA) to remove redundant sequences. Partial 16S rRNA gene sequences were compared with sequences deposited in GenBank (www.ncbi.nlm.nih.gov), Ribosomal Database Project II (www.rdp.cme.msu.edu), and EzTaxon. They were deposited in GenBank (Table 2). Phylogenetic trees were constructed by the neighbor-joining method by 1,000 bootstrap analysis using MEGA 4.

qPCR. The qPCR reaction mixture (25 μL) consisted of 1× SYBR Premix Ex Taq (II) (Takara), ROX Reference Dye II, group-specific primers (0.18 μM), MgCl2 (2 mM), and a DNA template (10 ng). The amplification program included an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 52.5–60 °C (Table 1) for 20 s, and extension at 72 °C for 45 s. Fluorescence data were collected at the end of each PCR cycle, and experiments were performed in triplicate using 7500 Fast Real-Time PCR (Applied Biosystems). Melting curve analysis were performed to determine the specificity of the resulting PCR products. Melting curves were obtained by slowly heating the sample from 60 to 95 °C at a rate of 0.1 °C/s with continuous fluorescence collection. The measured fluorescence was compared with the external standard, and the efficiency of amplification of each primer was tested along with the appropriate positive and negative controls. The type strains were used to construct a standard curve, and the results were converted into log values of the target microbial gene copy numbers per g of barley sample.

TaqMan PCR quantitative estimation of A. oryzae. Optimal primers and probes were designed based on the A. oryzae calmodulin gene by means of Integrated DNA Technologies software (www.idtdna.com). Reporter fluorescent dye 6-carboxyfluorescein (FAM) was labeled at the 5’ end, and conjugate BHQ1 was labeled at the 3’ end. Primers and probes were synthesized from Oligo (Macrogen, Seoul, Korea). The 1× Premix Ex Taq (Perfect Real Time; Takara) consisted of forward and reverse primers (0.18 μM), MgCl2 (2 mM), and the DNA template (10 ng). The amplification program included an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 52.5–60 °C (Table 1) for 20 s, and extension at 72 °C for 45 s. ROX II was used as reference dye.

Metabolite extraction and derivatization. Each barley nuruk starter sample was homogenized with a mortar and pestle for metabolomic analysis. The ground sample (100 mg) was extracted with 1 mL of 80% methanol.
methanol (Thermo Fisher Scientific, Fremont, CA) using an ultrasonicator (Hwashin, Seoul, Korea) for 30 min and centrifuged at 5,000 rpm for 10 min. Then 200 μL of the supernatant was dried completely with a speed vacuum concentrator (Biotron, Seoul, Korea) for 12 h. For oximation and derivatization, 50 μL of methoxyamine hydrochloride (10 mg/mL) in pyridine (Sigma-Aldrich, Seoul, Korea) was incubated with dried extract at 30 °C for 90 min. The resulting oximated samples were silylated with 50 μL of N-methyl-N-trifluoroorocacetamide containing 1% trimethylchlorosilane (Sigma-Aldrich, Seoul, Korea) at 37 °C for 30 min.

**GC-MS analysis.** A Varian CP-3800 gas chromatography system equipped with a Varian CP-8400 autosampler was coupled to a Varian 4000 ion-trap EI/CI mass spectrometric detector system (Varian, Palo Alto, CA). A VF-1MS capillary column (length 30 m × i.d. 0.25 mm × film thickness 0.25 μm) was used with helium at a constant flow of 1.0 mL/min. The derivatized sample (1 μL) was injected at a split ratio of 25:1. The oven temperature was held at 100 °C for 2 min, ramped to 300 °C at a rate of 5 °C/min, and held for 10 min. The mass data collected in EI mode using 70 eV of ionization energy were used for a full scan of m/z 50 to 1,000. The injector and transfer line temperatures were 200 and 250 °C respectively.

**Metabolite data analysis.** The GC-MS raw data were converted to netCDF (*.cdf) files with Xv capture (version 2.1; Adron Systems, Laporte, MN) and processed by XCMS for automatic peak detection and alignment to obtain a data matrix containing retention times, accurate masses, and normalized peak intensities. XCMS parameters were obtained using the R-program, version 2.12.2 (http://metlin-scripps.edu). The resulting data were exported to Excel (Microsoft, Redmond, WA), and statistical analysis was performed. After performing multivariate statistical analysis, major metabolites were positively identified using authentic standards by comparing the mass spectra and the retention times. When authentic standards were not available, tentative identification of compounds was made on the basis of MS spectra using the Wiley mass spectral database (Hewlett-Packard, 1995), NIST05 MS Library (NIST, 2005), and an in-house library.

**Statistical analysis.** Analysis of variance was done by ANOVA. Significant differences (p < 0.05) between means were determined by Student’s t-test. Multivariate data analysis was performed using SIMCA-P+ 11.0 (version 12.0; Umetrics, Umeå, Sweden). Data were centered and scaled using by unit variance (UV) scaling. Unsupervised principal component analysis (PCA), supervised partial least squares discriminant analysis (PLS-DA), and orthogonal projection to latent structures discriminate analysis (OPLS-DA) were then used to predict the variation of metabolites based on fermentation times. Metabolites with variable importance projection (VIP) values greater than 0.7 and p values less than 0.05 (threshold) were selected as metabolites to be used to discriminate the fermentation times of the barley nuruk samples. Culture dependent method results were obtained from the mean of three triplicates ± SD.

**Results and Discussion**

**Culturable microbes and identification**

Countable numbers of bacteria and fungi were obtained throughout the fermentation period except for zero days (excluded from the DNA-based studies). Rice nuruk samples are composed of mixed cultures containing filamentous fungi, yeast, and bacteria grown on cereals. Bacteria, molds, and yeasts have been identified in Chinese fermented liquor (Luzhou) both culture and non-culture methods.

The A. oryzae and Pantoea agglomerans identified by the main contributor to barley nuruk starter fermentation. The bacterial load steadily increased over 6 d, and then gradually decreased. In the total bacterial count, 5 log (p < 0.005) CFU increased from 3 to 6 d of fermentation (Fig. 1). In contrast, 1.4 log (p < 0.005) increased in A. oryzae over days 3 to 9. The pH of the fermentation medium remained nearly constant, without much fluctuation. The amount of viable A. oryzae was more or less maintained between 12 and 18 d of fermentation. Yu and co-workers have reported that Aspergillus was the most dominant fungus in rice wine

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**Table 2.** Predominant Bacteria and Fungi from Barley Nuruk Starter Identified by the PCR-DGGE Method

<table>
<thead>
<tr>
<th>Culture ID</th>
<th>Genbank access no.</th>
<th>Total bp</th>
<th>Closest relative</th>
<th>Next nearest GenBank ID</th>
<th>Maximum identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMNB2</td>
<td>JF510529</td>
<td>348</td>
<td>Pantoaea agglomerans strain ATCC 27987</td>
<td>FJ618824</td>
<td>100</td>
</tr>
<tr>
<td>FMNB5</td>
<td>JF510530</td>
<td>349</td>
<td>Enterobacter cloacae strain 6L</td>
<td>DQ919062</td>
<td>100</td>
</tr>
<tr>
<td>FMNB7</td>
<td>JF510531</td>
<td>349</td>
<td>Enterobacter cloacae bacterium N55S</td>
<td>ABS23725</td>
<td>100</td>
</tr>
<tr>
<td>FMNB3</td>
<td>JF510532</td>
<td>325</td>
<td>Pantoaea agglomerans; NCTC9381T</td>
<td>AJ251466</td>
<td>99</td>
</tr>
<tr>
<td>FMNB11</td>
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<td>Staphylococcus pasteuri strain ATCC51129</td>
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</tr>
<tr>
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<td>Staphylococcus warneri ATCC 2736(T)</td>
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<td>Bacillus subtilis strain PD-A10(T)</td>
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<td>FMNB F1</td>
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<td>Aspergillus niger</td>
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</tbody>
</table>

---

**Fig. 1.** Dynamics of Culturable Bacteria, Fungi, and Major Organisms in Barley Nuruk Starter Fermentation.

Values are means of three samples ± SD.
preparation whereas \( P. \) agglomerans was among the microorganisms present in the \( Laminaria \)-based nuruk medium. The microecological environments in the fermentation area, the shape and size of the container, the production process, and time were plays major role in the quality of mixed grain fermentation.

**Community analysis of barley nuruk starter by DGGE**

DGGE analysis of community DNA were performed from 3 to 18 d of fermentation. The bacteria and fungi identified on the basis of DGGE profiles were compared, and their nearest sequence matches are shown in Table 2. The bacterial sequences selected were deposited in GenBank under the following accession nos. JF510529 to JF510537. An abundance of \( A. \) oryzae and \( P. \) agglomerans was identified by PCR-DGGE band intensity, which was comparable with that of culture method results (Fig. 2). The cluster analysis of the DGGE profiles indicated a close relationship between fermentation time and the shift of microorganisms from time to time. Compared to conventional microbiological methods, analysis of community DNA followed by PCR-DGGE analysis showed significant differences in the profiles of the microbial communities. PCR-based methods have been found to be more rapid and reliable for detecting bacterial communities in Chinese liquor.

**PCR and sequencing of the 16S rRNA gene confirmed the identities of the microorganisms growing on barley nuruk starter.** They were restricted to \( Proteobacteria \), \( Firmicutes \), and \( Actinobacteria \). Approximately nine families were identified by these analyses. Based on the DGGE profile, limited bacterial profiles between 3 and 6d of fermentation were observed and, they subsequently decreased. A similar profile was reported for barley-based fen liquor fermentation. Only two DNA bands were detected over the 18d fermentation, and only \( P. \) agglomerans was prominent throughout the fermentation process, with varying degrees of DGGE band intensity. The \( P. \) agglomerans bands suggest that this phylotype was dominant throughout barley nuruk fermentation. During the first 6d of fermentation, bacterial richness was high, but subsequently decreased. Routine culture methods identified only three genera (\( Bacillus \), \( Acetobacter \), and \( Arthrobacter \)) in the \( Zaopei \) that produces \( Luzhou \)-flavored liquor, but other genera were identified by the PCR-DGGE method. Nearly 14 genera were identified but most of them were unculturable microorganisms. In theory, molecular methods can detect more genera and species, including cultured and uncultured microorganisms, perhaps due to the choice of primers. Group-specific primers can provide more detailed microbial profiles than a single pair of primers.

**Microbial diversity versus time points**

The DGGE profiles of the bacteria and the fungi were dissimilar during fermentation. On day 6, greater bacterial diversity was observed, whereas higher fungal diversity was detected on day 9. Only the \( A. \) oryzae was most abundant, identified in both culture dependent and culture independent methods (Fig. 3). It is controversial with the earlier report of rice traditional starter, whereas the \( Aspergillus \) load was gradually fell down and finally diminished on day 16. \( Saccharomyces fibuliger \), \( Saccharomycopsis cerevisiae \), and \( Lichtheimia \) were also detected. They have rarely been reported in few

**Fig. 2.** Community Analysis of Barley Nuruk by DGGE. 
(a) Comparison of DGGE band intensities of \( Aspergillus \) oryzae and \( Pantoea \) agglomerans; b, qPCR results for genes representing total bacteria, fungi, and \( Aspergillus \) oryzae expressed during fermentation.

**Fig. 3.** Phylogenetic Comparison of 26S rRNA Sequences of Molds and Yeasts Isolated from Barley Nuruk Fermentation. Sequences from GenBank are indicated by accession numbers. Main clades and differences between parsimony are represented by numbers.
fermented grains. Saccharomycopsis spp., such as S. fibuligera, are commonly found in Southeast Asian rice wine starter.

qPCR estimation of total bacteria and fungi

In this study, differences between the amounts of fungi and bacteria during the fermentation process were observed. The total bacterial load increased during the first 6 d of incubation and then decreased, while the fungal load increased maximum. Fungal growth can lower the pH of the media slightly and might have been one of the reasons for reduced bacterial growth. Since A. oryzae was a major contributor to barley nuruk starter, it was attempted to quantify in the fermented barley samples. An A. oryzae spore-spiked barley experiment showed the minimum for DNA extraction and it was determined to be $9.2 \times 10^3$ conidia per g of sample.

Aspergillus oryzae was predominant among the fungi (8.07 conidia/g) and increased by 4% ($p < 0.001$) on day 3 of fermentation (Fig. 2). Most fungal communities are more oxygen-, temperature-, and pH-dependent. A. oryzae can provide adequate nutrition for fungal growth, and its metabolism may play a role in further saccharification. Barley-carbohydrates can be used as substrates for energy and the synthesis of fatty acids. Most studies have reported that Aspergillus is the genus responsible for starch saccharification in nuruk, in agreement with previous reports on koji and Zaopei.

Multivariate analysis of barley nuruk based on fermentation time

The metabolic pattern of barley nuruk starter varies with fermentation time. The metabolites in the initial (0–6 d) and later stages (9–18 d) of fermentation were separated along principal component 1 (PC1, 53.3%) in the PCA score plot (Fig. 4a), but there were no significant differences during the later stage, after 12 d of fermentation. Similar patterns were found by PLS-DA analysis (Fig. 4b). The significant variations in metabolites based on fermentation times are summarized in Table 3. Organic acids (phosphoric acid, malic acid, succinic acid, and citric acid), amino acids (glutamine, aspartic acid, and glutamic acid), sugars (xylose, fructose, glucose, galactose, sucrose, lactose, and maltose), and sugar alcohols (glycerol, arabitol, mannitol, and xylitol) were major metabolites found during the fermentation of barley nuruk. Aspergillus scarifies the carbohydrate for energy metabolism, and can be utilized by other microbial communities. Malic acid, fructose, glucose, galactose, sucrose, lactose, and maltose were found mainly at the early stage of fermentation (0–6 d), while succinic acid, citric acid, glutamine, aspartic acid, glutamic acid, xylose, mannitol, xylitol, linoleic acid, and oleic acid were responsible for the later stage (9–18 d).
Metabolite changes in barley nuruk during fermentation by bacteria and fungi

Representative fermentation stages (6 and 9 d) were selected based on the microbial community profile from DGGE, and were used to compare the different microorganisms and metabolite changes by OPLS-DA. An OPLS-DA score plot of the two partial least squares components, OPLS 1 and OPLS 2, clearly showed a separation of the two fermentation stages (Fig. 5). The initial stage of fermentation (0–6 d), disaccharides such as sucrose, lactose, and maltose decreased, while monosaccharides such as glucose and fructose increased (Fig. 6). This suggests that disaccharides were converted to monosaccharides by the bacterial enzymes.\(^\text{23}\) In general, sugars such as glucose, fructose, galactose, and sucrose decrease at the later stage of fermentation. A major reason for the reduction in sugars during fermentation is that sugars are used as substrates for microbial growth and organic acid production.\(^\text{24}\) Sugar alcohols, and sugar derivatives increased during fermentation in this study. The 6 d fermentation was enriched with phosphoric acid, succinic acid, malic acid, citric acid, glucose, fructose, galactose, sucrose, lactose, maltose, glycerol, and arabinol, while 9 d fermentation was characterized by enrichment in glutamic acid, aspartic acid, glutamic acid, xylose, mannitol, xylitol, linoelie acid, and oleic acid.

Microbial shift-dependent metabolite changes contributed to the appearance of the fermentation patterns

The PCR-DGGE and qPCR results showed a major shift in the amounts of bacteria and fungi during day 6 and 9 of fermentation respectively. The metabolite profiles at these two time points were analyzed using PLS-DA models in order to study the effects of microbial fermentation. The PLS-DA score plot of two partial least square components, PLS 1 and PLS 2 were showed separated metabolite profiles at day 6 and 9 (Fig. 5). Glutamine, glycerol, malic acid, aspartic acid, glutamic acid, mannitol, fructose, glucose, xylitol, sucrose, and maltose were observed as major differential metabolites. On day 6 of fermentation, a box-and-whisker plots showed that the cultures were enriched in glycerol, malic acid, fructose, glucose, sucrose, and maltose. Higher glucose levels might have been the reason for the proliferation of bacteria on day 6. Bacteria belonging to the genera Pantoaea and Enterobacter were observed at the beginning of fermentation. P. agglomerans and Enterobacter cowanii are capable of decomposing organic substances and increasing acids and sugar alcohols.\(^\text{25}\) However, at day 9, the cultures were enriched in glutamine, aspartic acid, glutamic acid, mannitol, and xylitol (Fig. 6). Other identified microbes, S. fibuligera are well-known producers of α-amylase and glucoamylase, which can convert glucose.\(^\text{26}\)
Aspergillus oryzae was a major contributor to barley nuruk fermentation.

After 6 d of incubation, the total amount of bacteria decreased, and fungi were the main microorganisms during the later stages of fermentation. Fungi utilized the readily available sugars and amino acids and increased A. oryzae biomass, as evident in the qPCR results. Amino acids, such as glutamine and aspartic acid, are often reported for fungal fermentation. The amounts of sugar alcohols, such as mannitol and xylitol, increased during later fermentation stage (9–18 d). Most of A. oryzae contributed to the initial production of amylase and glucoamylase. Amylase hydrolyzes the 1,4-α-glucosidic linkage in polysaccharides, yielding dextrins, oligosaccharides, and glucose, whereas glucoamylase hydrolyzes both 1,4-α- and 1,6-α-glucosidic linkages in polysaccharides, producing increased amounts of glucose. Several yeasts and fungi are involved in sugar alcohol conversion in various biosynthetic pathways. Saccharification and protein digestion by amylolytic and proteolytic enzymes have been reported for nuruk fungi. In this study, the relative contents of amino acids and fatty acids were observed to increase gradually over 15 d, whereas malic acid and disaccharides (sucrose, lactose, and maltose) decreased over 12 d (Fig. 6). Similarly to soyabean fermentation, the barley proteins were degraded and glutamic acid, glutamine and aspartic acid by the microbial community enzymes. Most of the sugars, including glucose, fructose, galactose, sucrose, lactose, and maltose were decreased up to 12 d and slightly improved during the later stage of fermentation. Reduction in sugars during fermentation is mainly due to sugars being used as substrates for microbial growth and organic acid production. In the present study, succinic acid and citric acid gradually higher over 15 d of fermentation. Cheung and co-workers reported that the production of organic acids inhibited microbial growth, which might have been the reason why the total amount of fungi and bacteria in barley nuruk samples decreased after 9 d in the present study.

Previous studies of various methods of nuruk fermentation focused on microbial fermentation or alcohol production. In the present study, barley nuruk starter microbial dynamics and their metabolome were compared by the PCR-DGGE, qPCR, and GC-MS methods. Our DGGE community results indicate that more than one type of fungi and bacteria are present in traditional barley nuruk starter. A. oryzae was predominant at 9 d of fermentation, and remained such until the end of the fermentation even in the presence of other genera. Organic and amino acids, sugars, and sugar alcohols can originate from both microbial metabolism and conversion from raw materials. The present study indicates that
a mixture of bacterial and fungal species contributes to the saccharification of barley nuruk samples and varies at different times. The correlation between microbes and the metabolome of barley nuruk may aid in to understanding and controlling the fermentation process, but traditional starter fermentation varies in microbial consortium and metabolites from different fermentation boxes and different places. The sampling method and more precise analysis methods must be identified to overcome these pitfalls.

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