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Short communication: Physicochemical features and microbial community of milk kefir using a potential probiotic *Saccharomyces cerevisiae* KU200284

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ABSTRACT

The aim of this study was to analyze the β-glucan contents, physicochemical features, and microbial communities in milk kefir prepared using *Saccharomyces cerevisiae* KU200284 isolated from cucumber *jangajji*, a fermented vegetable commonly eaten in Korean. Three types of milk kefir were manufactured, with (1) activated kefir grain, (2) activated kefir grain with commercial *S. cerevisiae* BOF, and (3) activated kefir grain with *S. cerevisiae* KU200284. β-Glucan contents of milk kefir using kefir grain and kefir grain with *S. cerevisiae* strains BOF and KU200284 were 8.29, 8.59, and 8.57%, respectively. The pH, titratable acidity, viscosity, Brix level, and alcohol contents of milk kefir using kefir grain with *S. cerevisiae* strains were acceptable compared with milk kefir using only kefir grain. In milk kefir produced using kefir grains and *S. cerevisiae* strains, 16S rRNA reads showed representative strains of *Lactobacillus kefiranofaciens* (>72% relative abundance) and *Acetobacter fabarum* (>16% relative abundance). In particular, milk kefir using kefir grain with *S. cerevisiae* KU200284 had the highest relative abundance of *L. kefiranofaciens*. In addition, the internal transcribed sequence (ITS) rRNA reads in tested milk kefir showed representative strains of *Kluyveromyces marxianus* (>52% relative abundance) and *Saccharomyces cerevisiae* (>16% relative abundance). In contrast, milk kefir using *S. cerevisiae* strains had higher relative abundance of *S. cerevisiae* (>37%). The β-glucan production, physicochemical properties, and microbial community profiling indicate that *S. cerevisiae* KU200284 could be used in functional dairy products as a starter culture.

Key words: *Saccharomyces cerevisiae*, milk kefir, physicochemical feature, microbial community

Short Communication

Kefir is a fermented dairy beverage made from kefir grains, including lactic acid bacteria, yeasts, and acetic acid bacteria (Garofalo et al., 2015). Kefir grains are elastic, slimy, white to light yellow, and with a small, irregular, round shape, often differing in size, and sometimes resembling a mushroom (Kabak and Dobson, 2011). In general, kefir grains are composed of *Kluyveromyces*, *Saccharomyces*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Acetobacter* species (Prado et al., 2015). Kefir is obtained through the lactic or alcoholic fermentation of milk, and their microbiota produces bioactive compounds, including peptides, amino acids, ethanol, CO_2, acetaldehyde, acetoin, diacetyl, exopolysaccharides, folic acid, calcium, vitamins (B_1, B_{12}, and K) (Kabak and Dobson, 2011; Garofalo et al., 2015). Functional effects have been also reported, including immune system modulation, enhanced digestive health, and antimicrobial, antitumor, and antioxidant activities (Farnworth, 2005).

Saccharomyces cerevisiae has been classified as QPS (qualified presumption of safety) according to the European Food Safety Authority (Leuschner et al., 2010). *Saccharomyces cerevisiae* has been used in winemaking, baking, and brewing, with reported functions including the production of β-glucan, glutathione, glutathione derivatives, protein, fiber, vitamin B, and folic acid derived from cell wall (Hong et al., 2019). *Saccharomyces cerevisiae* or *Saccharomyces boulardii* has mainly been used as a probiotic for treatment of antibiotic-associated diarrhea, and studies have indicated that either species can perform immunomodulatory, gastrointestinal modulatory, and antioxidant functions (Fakruddin et al., 2017). *Saccharomyces cerevisiae* KU200284 was previously isolated from cucumber *jangajji*, a Korean vegetable fermented food. This strain was demonstrated to have the highest β-glucan production, stability in artificial gastric conditions, without transfer of antibiotic resistance genes, and protection against DNA damage among the tested *S. cerevisiae* strains (Lee et al., 2019). In addition, *S. cerevisiae* KU200284 demonstrated the highest adhesion to intestinal cells. Therefore, we made

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milk kefirs using kefir grains, kefir grains with *S. cerevisiae* BOF, and kefir grains with *S. cerevisiae* KU200284, and evaluated their physicochemical features. In addition, we performed a next-generation sequencing-based microbiome analysis to assess the microbial community composition of milk kefir inoculating potential probiotic yeast strains.

We used *S. cerevisiae* KU200284 or *S. cerevisiae* BOF isolated from a pharmaceutical product (Biocodex, Gentilly, France) as starter culture supplement. Yeast strains were cultured in yeast malt (YM) medium (Difco, Detroit, MI) at 25°C for 48 h for further study.

The kefir grains were obtained from a local household (Lyosan, Canada). Activation of kefir grains followed the method of Leite et al. (2013) with modification. Two grams of kefir grains was used to inoculate 100 mL of sterilized milk and then incubated at 26°C for 24 h. After incubation, the culture was filtered through a plastic strainer to remove coagulated milk and gently washed with sterilized water. This step was repeated 4 times until the kefir grains had appropriate characteristics and their biomass had increased by 10%.

Three types of milk kefir were manufactured using activated kefir grains. Milk kefir was inoculated with 2% (wt/vol) activated kefir grains into sterilized whole milk (**M1**). Sterilized whole milk was inoculated with 2% activated kefir grains and 6 log cfu/mL of either *S. cerevisiae* BOF (**M2**) or *S. cerevisiae* KU200284 (**M3**). The inoculated milk was fermented at 26°C for 24 h, and sampling was performed at 0, 8, 16, and 24 h for physicochemical characterization.

At 24 h of sampling, the milk kefirs were freeze-dried, and dried samples were quantified using the Megazyme kit (Megazyme Inc., Wicklow, Ireland) following the manufacturer's guideline for glucan calculation.

The pH of milk kefir samples was measured using a pH meter (SevenCompact, Mettler Toledo, Greifensee, Switzerland), and titratable acidity was measured using a titrator (848 Titrino Plus, Metrohm, Herisau, Switzerland). The viscosity of kefir samples was measured 3 times at 1-min intervals from 2 to 4 min at 30 rpm, using a DV-E viscometer (Brookfield Engineering Laboratories Inc., Middleboro, MA) 64 spindle. The Brix values of kefir samples were measured using a Brix meter (PR-1, Atago, Tokyo, Japan) in the range of 0 to 32% Brix. The alcohol contents of kefir beverages were measured using a concentration meter (DMA 4500A, Anton Paar GmbH, Graz, Austria).

For microbial composition, total DNA was extracted from 30 mL of milk kefir after 24 h of fermentation, using a PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the manufacturer's protocol. The DNA quality was measured by PicoGreen dye (Invitrogen, Carlsbad, CA) and Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The V3–V4 regions of bacterial 16S rRNA genes were amplified by PCR from the genomic DNA (forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGGACAGCCTACGGGNGGCWGCAG-3′; reverse primer: 5′-GGCTCGGATGTGTATAGAACAGGACTACHVGGGTATCTAATCC-3′). The internal transcribed sequence (**ITS**) rRNA genes were used for fungal microbiome analysis (forward primer: 5′-GCATCGATGAAGAACGCAGC-3′; reverse primer: 5′-TCCTCCGCTTATTGTATGC-3′). The final products were normalized and pooled using the PicoGreen kit. Library size was verified using the LabChip GX HT DNA high sensitivity kit (PerkinElmer, Waltham, MA). Sequence processing was performed using the Illumina MiSeq platform (Illumina Inc., San Diego, CA). Sequence data were trimmed using the FASTX tool (v 0.0.14) and analyzed with QIIME 1.8.0 (Caporaso et al., 2010). The sequences were clustered into operational taxonomic units (**OTU**) using the default UCLUST closed-reference OTU picking algorithm (pick_closed_referne_otus.py) against the curated sequences (Kim et al., 2019); OTU represented >0.5% of the total sequences.

The results are expressed as mean and standard deviation of 3 replicates in triplicate. One-way ANOVA was used to determine significant differences. Comparison of means was carried out using Duncan's multiple range test ($P < 0.05$).

Saccharomyces cerevisiae KU200284 was selected as a kefir starter with a view to its production of bioactive compounds and potential probiotic properties (Lee et al., 2019). The β-glucan contents of M1, M2, and M3 were 8.29, 8.59, and 8.57%, respectively (Table 1), and did not differ significantly between samples. However, kefir with *S. cerevisiae* strains (M2 and M3) showed numerically higher β-glucan contents. The total glucan contents of samples M1, M2, and M3 were 9.18, 9.18, and 9.46%, respectively (Table 1).

Changes in pH and titratable acidity values of milk kefirs during fermentation are shown in Figure 1A. Ini-

Table 1. Glucan contents of the milk kefir[1,2]

Item	M1	M2	M3
Total glucan (%)	9.18 ± 0.99[b]	9.18 ± 0.50[ab]	9.46 ± 0.00[a]
α-Glucan (%)	0.89 ± 0.00[a]	0.59 ± 0.51[a]	0.89 ± 0.00[a]
β-Glucan (%)	8.29 ± 1.00[b]	8.59 ± 0.02[a]	8.57 ± 0.00[a]

[a,b]Means with different superscript letters within a row indicate statistical differences ($P < 0.05$).

[1]Results are represented as mean ± SD.

[2]M1 = kefir grains (2%); M2 = kefir grains (2%) + *Saccharomyces cerevisiae* BOF; M3 = kefir grains (2%) + *Saccharomyces cerevisiae* KU200284.

tial pH of milk kefir ranged from 6.68 to 6.73, and final pH ranged from 4.90 to 4.16 at 24 h of fermentation. In contrast, acidity increased from 0.13 to 1.60% during the 24-h fermentation. Generally, kefir beverages reach a pH of 4.4 to 4.6 (Wszolek et al., 2001) and acidity of 0.97 to 1.40% (Kim and Cho, 2009).

Changes in viscosity and Brix values of milk kefir during fermentation are shown in Figure 1C and 1D. All samples showed similar viscosity and Brix values to those at 8 h of fermentation. At 24 h, M2 had the lowest viscosity of 1,212 cP, and M1 the highest viscosity of 1,948 cP; M2 showed the lowest Brix value and M1 the highest Brix value.

Changes in alcohol contents of milk kefir during fermentation are shown in Figure 1E. All samples showed 0% until 8 h; M1, M2, and M3 showed 0.29, 0.64, and

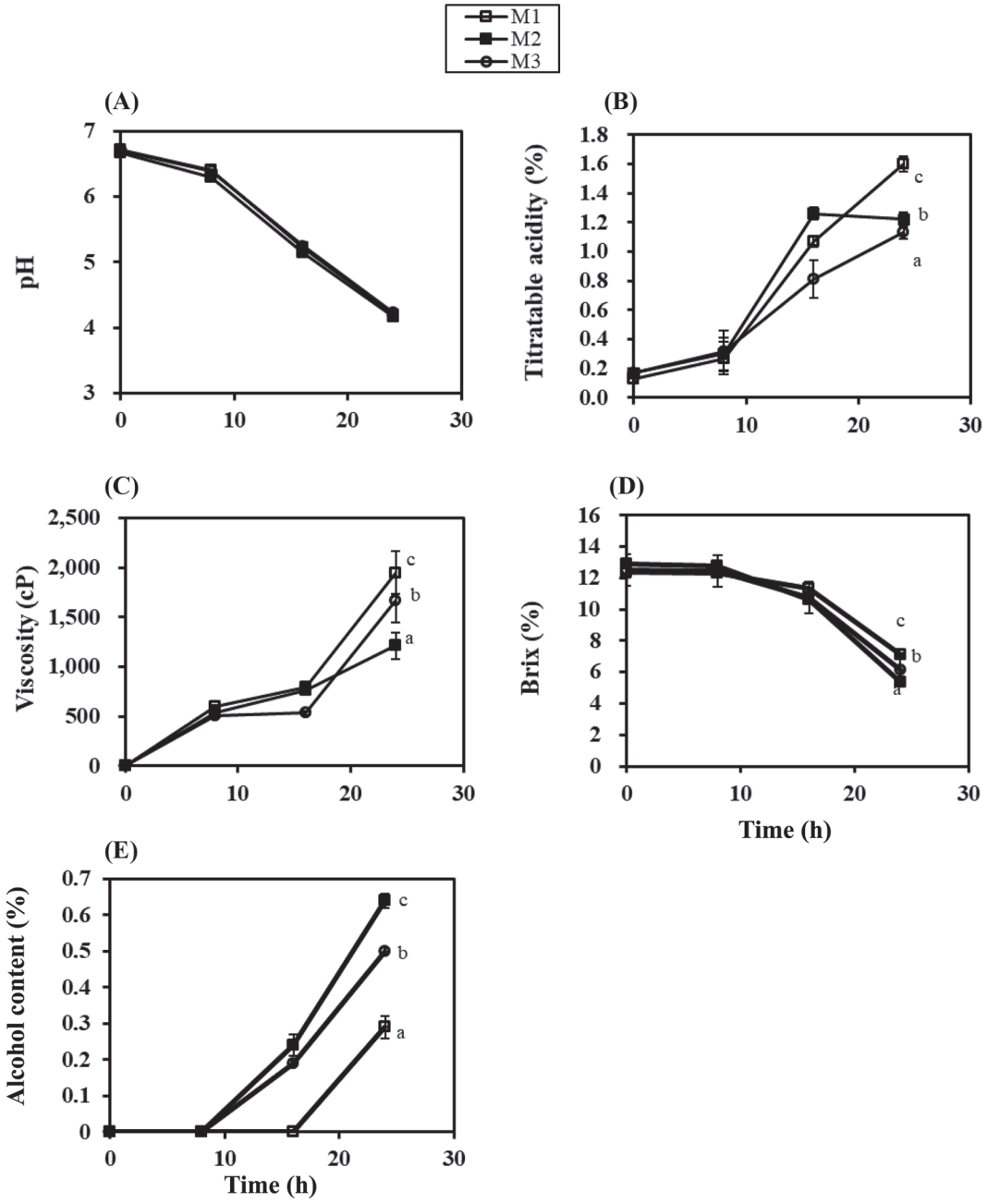

Figure 1. Changes in (A) pH, (B) titratable acidity, (C) viscosity, (D) Brix, and (E) alcohol content in kefir beverages made with kefir grains (M1; 2%); kefir grains (2%) + *Saccharomyces cerevisiae* BOF (M2); kefir grains (2%) + *Saccharomyces cerevisiae* KU200284 (M3). Different letters (a–c) represent significant differences between values ($P < 0.05$). Results are presented as mean ± SD.

0.5% alcohol at 24 h of fermentation, respectively. The difference in alcohol contents was dependent on inoculated *S. cerevisiae* strains. The alcohol contents of kefir beverages are reported to vary from 0.5 to 1.5% (Marshall and Cole, 1985). Alcoholic fermented milk has been reported as a high-value functional food, with functions such as inhibition of intestinal harmful bacteria and flavor (Prado et al., 2015).

Kefir beverage is a natural probiotic carrier, consumed for its positive healthy effects (Koh et al., 2017). Therefore, the microbial community of kefir was determined using 16S rRNA and ITS sequencing. In the 16S rRNA sequence, read counts of M1, M2, and M3 milk kefir were 83,871, 89,673, and 111,114. The number of observed OTU, evaluated on a 97% similarity threshold by the Chao1 estimator, ranged from 11 to 12 OTU. The rarefaction curves of observed species richness reached a saturation phase, with a 97% sequence similarity cut-off. The Shannon diversity and Simpson's indices ranged from 0.94 to 1.07 and 0.36 to 0.42, respectively. Good's coverage was estimated to evaluate the completeness of sampling, and samples M1, M2, and M3 had values of 0.99, 0.99, and 1.00, respectively.

The 16S rRNA reads from all samples were assigned to 7 species, including *Lactobacillus kefiranofaciens* and *Acetobacter fabarum* (Table 2). The sample of M1 contained *L. kefiranofaciens* (76.65%), *A. fabarum* (19.94%), and *Lactobacillus kefiri* (3.11%); M2 contained *L. kefiranofaciens* (72.70%), *A. fabarum* (22.07%), and *L. kefiri* (4.85%); M3 contained *L. kefiranofaciens* (78.34%), *A. fabarum* (16.91%), and *L. kefiri* (4.51%). *Lactobacillus kefiranofaciens* has a reported abundance of 97.63% in Turkish kefir grains using metagenomic analysis (Nalbantoglu et al., 2014). *Lactobacillus kefiranofaciens* produced "kefiran (water-soluble polysaccharide)" (Wang et al., 2012; Garofalo et al., 2015). In addition, *Lactobacillus* strains showed the highest abundance in M3 milk kefir at the genus level. *Lactobacillus buchneri*, *Lactobacillus crispatus*, and *Lactobacillus psittaci* were identified as minor microorganisms of milk kefir.

For the ITS rRNA sequence, each sequence read of M1, M2, and M3 milk kefirs was 163,775, 190,149, and 191,918, respectively (data not shown). For ITS rRNA reads, the numbers of observed OTU were 9, 8, and 47, respectively, in M1, M2, and M3. The Shannon diversity and Simpson's indices ranged from 1.12 to 1.24 and 0.43 to 0.53, respectively. Good's coverage of samples M1, M2, and M3 were 1.00, 0.99, and 0.99, respectively.

The ITS rRNA reads from all samples were assigned to 19 species, including *Kluyveromyces marxianus* and *S. cerevisiae* (Table 2); *K. marxianus* was dominant in all samples, particularly M1, where it was present at 72.559%. Kefir samples M2 and M3 contained 52.155 and 57.390%, respectively. The relative abundance of *S. cerevisiae* was higher in M2. *Kluyveromyces marxianus* has been reported to be the dominant yeast in Chinese koumiss and *Lactobacillus* fermented milk (Ni et al., 2007; Zhang et al., 2017). A mixed culture of *K. marxianus* produced bioactive peptides with antibacterial, antihypertensive, and other properties (Zhang et al., 2017). The samples of M2 and M3 contained a higher *S. cerevisiae* proportion than M1, which was not inoculated with *S. cerevisiae*.

In this study, we produced milk kefir using potential probiotic strain *S. cerevisiae* KU200284. In terms of physicochemical features, the addition of *S. cerevisiae* decreased viscosity, Brix values, and total acidity and increased glucan and alcohol contents. In the microbial community, *L. kefiranofaciens*, *A. fabarum*, *K. marxianus*, and *S. cerevisiae* were dominant in all milk kefir samples. These strains could have important roles during kefir fermentation. In particular, M3 kefir had the highest abundance of *Lactobacillus* spp. and *Saccharomyces* spp. of the tested milk kefir. Therefore, *S. cerevisiae* KU200284 could be used as a starter culture for kefir production using a probiotic carrier.

Table 2. Relative abundance analysis by 16S rRNA and internal transcribed spacer (ITS) rRNA sequence

Taxon	Relative abundance[1] (%)		
	M1	M2	M3
16S rRNA sequence			
Lactobacillus kefiranofaciens	76.646	72.696	78.343
Acetobacter fabarum	19.935	22.072	16.908
Lactobacillus kefiri	3.113	4.851	4.505
Gluconobacter cerevisiae	0.207	0.101	0.081
Lactobacillus buchneri	0.063	0.101	0.081
Lactobacillus crispatus	0.020	0.023	0.018
Lactobacillus psittaci	0.010	0.004	0.012
ITS rRNA sequence			
Kluyveromyces marxianus	72.559	52.155	57.390
Saccharomyces cerevisiae	16.669	44.431	37.486
Other	10.687	3.387	4.825
Penicillium sp.	0.001	0.002	0.079
Fungi	0.000	0.000	0.007
Dokmaia sp.	0.000	0.000	0.013
Periconia macrospinosa	0.000	0.000	0.008
Saitozyma podzolica	0.000	0.000	0.006
Chaetothyriales sp.	0.000	0.000	0.018
Bipolaris sorokiniana	0.000	0.000	0.002
Aspergillus sp.	0.001	0.001	0.004
Capnodiales sp.	0.000	0.000	0.002
Drechslera salviniae	0.000	0.000	0.003
Helotiales sp.	0.000	0.000	0.003
Pleosporales sp.	0.000	0.000	0.002
Talaromyces aculeatus	0.002	0.000	0.004
Teratosphaeriaceae sp.	0.000	0.000	0.002
Tremellales sp.	0.000	0.000	0.003
Pichia fermentans	0.081	0.022	0.017

[1]M1 = kefir grains (2%); M2 = kefir grains (2%) + *Saccharomyces cerevisiae* BOF; M3 = kefir grains (2%) + *Saccharomyces cerevisiae* KU200284.

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