Polyphasic Microbial Analysis of Traditional Korean Jeung-Pyun Sourdough Fermented with Makgeolli

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Introduction

Fermented foods are produced worldwide using a variety of raw materials, microorganisms, and techniques. Sourdough products provide several health benefits, such as increased mineral bioavailability, reduced gluten intolerance, and a lower postprandial glucose response [1]. However, wheat is the dominant cereal used for sourdough products throughout the world, and many people have allergies to wheat, barley, and rye glutens [2]. Thus, there is a need to explore other cereal-based sourdoughs that have no negative effects on human health.

Rice is as a staple food in most Asian countries, and no side effects have been reported so far for rice-based sourdoughs [3]. Jeung-pyun is a fermented rice cake produced by fermenting rice sourdough using makgeolli, a traditional Korean rice wine, in the presence of yeast and lactic acid bacteria (LAB). The goal of this study was to conduct biochemical and microbial analyses of five different rice sourdoughs, each fermented with a different commercial makgeolli, using culture-dependent and culture-independent approaches. All sourdough samples fermented with different makgeolli for 6.5 h showed different profiles in pH, total titratable acidity, organic acid concentration, and microbial growth. LAB belonging to different genera were identified based on colony morphology on modified MRS and sourdough bacteria agar medium. PCR-denaturing gradient gel electrophoresis analyses of the five sourdoughs showed different bands corresponding to LAB and yeast. 16S/26S rRNA gene sequence analyses of the samples confirmed that the predominant LAB in the five fermented rice doughs was Lactobacillus plantarum, Lb. pentosus, and Lb. brevis. Various other Lactobacillus spp. and Saccharomyces cerevisiae were common in all five fermented samples. This study provides comprehensive and comparative information on the microflora involved in fermentation of rice sourdough and signifies the need to develop effective starters to enrich the quality of jeung-pyun.

Keywords: Jeung-pyun, makgeolli, sourdoughs, lactic acid bacteria
carbon dioxide, organic acids, and exopolysaccharides. They also produce several enzymes that help in hydrolyzing complex polysaccharides to simple sugars, thus modifying the environment and promoting the growth of other LAB belonging to different genera at later stages [8]. Hence, analysis of the different microorganisms involved in the fermentation of sourdough is critical for the development of products in the food industry.

In the present study, we performed polyphasic analysis of the microbial community in jeung-pyun fermented with makgeolli. Five commercial makgeolli produced in Korea were used as starters for fermentation of the rice doughs, and the respective sourdough samples were subjected to culture-dependent and culture-independent analyses to identify the predominant microorganisms. The byproducts produced by these microbes were also analyzed. The present study will facilitate the selection of microbes with desired qualities to be used as starter cultures for the production of rice-based sourdough products with improved quality, texture, and shelf life.

Materials and Methods

Jeung-Pyun Dough Preparation

Jeung-pyun doughs were prepared by mixing 100 g of rice powder, 40 ml of water, 30 ml of commercial makgeolli products (makgeolli a, makgeolli b, makgeolli c, makgeolli d, or makgeolli e), 25 g of sucrose, and 1.0 g of NaCl. The doughs were mixed with a hand mixer (model JU07408-4001; Sanshui Hop Shin Metal & Plastic Manufactury Ltd, Japan) for 3 min and fermented at 35°C for 6.5 h.

Measurement of pH and Total Titratable Acidity (TTA) of the Fermented Rice Doughs

An aliquot of 10 g of fermented rice dough was blended with 90 ml of distilled water and the pH was measured. To determine the TTA, the suspension was titrated against 0.01 N NaOH using phenolphthalein as the indicator. The TTA was expressed as percent lactic acid [4].

Enumeration and Isolation of LAB and Yeast in Fermented Rice Doughs

Ten grams of fermented rice dough was homogenized with 90 ml of sterile NaCl solution (0.85% (w/v)) and the suspension was serially diluted (10^{-1} to 10^{-6}) using NaCl solution. For counting and isolation of LAB and yeast, 20 μl of each dilution was plated on de Man, Rogosa, and Sharpe (MRS) agar with bromphenol blue (BPB), and on sourdough bacteria (SDB) agar with BPB (Difco, USA), respectively, and incubated at 30°C for 2 days [9]. The composition of BPB-MRS media was as follows: 10 g/l proteose peptone, 10 g/l beef extract, 20 g/l glucose, 1 g/l Tween-80, 2 g/l ammonium citrate, 5 g/l sodium acetate, 0.1 g/l magnesium sulfate, 0.005 g/l manganese sulfate, 2 g/l dipotassium phosphate, 5 g/l yeast extract, 0.02 g/l bromphenol blue, and 5 g/l cycloheximide. The composition of BPB-SDB media comprised 3 g/l yeast extract, 0.02 g/l bromphenol blue, 5 g/l cycloheximide, 20 g/l maltose, 6 g/l trypticase, 3 g/l Tween-80, and 10 g/l fresh yeast extract (FYE). FYE was prepared by autoclaving 20% of commercial baker’s yeast (Saccharomyces cerevisiae) in distilled water for 30 min at 15 psi. The suspension was settled overnight at 8°C, and the supernatant was clarified by centrifugation. Colonies were independently picked from each plate for analysis of morphology mediated by change in color from blue to white due to production of organic acids by LAB.

Measurement of Organic Acids and Water-Soluble Free Sugars in Fermented Rice Doughs

Ten grams of jeung-pyun dough was blended with 90 ml of distilled water and the suspension was centrifuged at 13,000 × g for 10 min. The supernatant was boiled for 5 min and centrifuged again. The resulting clear broth was filtered through a 0.2 μm syringe filter. The filtrate was used for analysis of different sugars (glucose, fructose, sucrose, and maltose) by high-performance liquid chromatography (HPLC) (Acme 9000; Young Lin Instrument Co., Korea) using an Asahipak NH2P-50 4E column (Shodex, Japan) with a refractive index detector (Young Lin Instrument Co.). Acetonitrile and water (80:20 (v/v)) were used as the mobile phase at a flow rate of 1.0 ml/min. Lactic acid and acetic acid were measured by HPLC using an Aminex HPX-87H column (Bio-Rad, USA). H2SO4 (0.008 N) was used as the mobile phase at a flow rate of 0.6 ml/min.

Extraction and PCR Amplification of DNA from Fermented Rice Doughs

Genomic DNA was extracted from the five doughs fermented with different makgeolli using genomic DNA Prep kits for bacterial and yeast cells (SolGent, Korea) [10]. Total bacterial DNA was used as a template for PCR amplification of the V3 region of the 16S rRNA gene using the universal primers 338F and 518R. Yeast DNA was used as template for PCR amplification of the 28S rRNA gene using the universal primers GC-NL1F and LS2R [11]. The PCR products were confirmed by electrophoresis in 1% agarose gel.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

The PCR-amplified 16S rRNA gene of bacteria and the 28S rRNA gene of yeast were analyzed by DGGE with a DCode system (Bio-Rad) [12]. The PCR products were applied to 8.0% (v/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels in 1× Tris-acetate-EDTA buffer. Parallel electrophoresis experiments were performed at 60°C using gels containing a 30% to 60% (v/v) urea-formamide denaturing gradient (with 100% (v/v) corresponding to 7 M urea and 40% (v/v) formamide) increasing in the direction of electrophoresis. The gels were subjected to electrophoresis for 30 min at 20 V and then 16 h at 60 V, stained with ethidium bromide, and visualized under UV light.
Sequencing of DGGE Bands
The gel bands were excised and incubated overnight in 20 µl of Tris-EDTA buffer, and the DNA was recovered. For DNA sequence analysis, PCR re-amplification was performed with the same primers (without the GC clamp). The re-amplified PCR products were purified with a DNA purification kit (SolGent) according to the manufacturer’s protocol and sequenced by SolGent Co., Ltd. The sequences were then compared with the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, USA) [13].

Results

Fermentation Profiles of Rice Sourdoughs
The pH, TTA, and viable cell count of dough samples made with the five different makgeolli were monitored. The pH decreased during fermentation in all samples (Fig. 1A). The maximum pH drop was observed in sample b (final pH of 4.2). The final TTA values for the five samples ranged between 0.54% (sample d) and 0.81% (sample a) (Fig. 1B). The cell count results were in accordance with the pH and TTA results (Fig. 1C): the highest viable cell count was observed in sample b (8.2 Log CFU/g) and the lowest was observed in sample d (5.2 Log CFU/g).

Organic Acid Content of Rice Sourdoughs
Apart from their antimicrobial properties, organic acids are the key components that determine the flavor and shelf-life of fermented products. The amounts of acetic acid and lactic acid and their ratio were significantly different among the five jeung-pyun doughs produced with different makgeolli (Fig. 2). The total organic acid content after 6.0 h of fermentation was highest in sample c and lowest in sample d (40.87 and 21.53 mmol/kg, respectively). Apart from lactic acid, a significant amount of acetic acid was also produced in the fermented rice dough samples (Fig. 2), indicating the presence of heterofermentative LAB in all makgeolli used for the experiment. In samples a, b, and e, the amount of acetic acid produced was very high compared with lactic acid, and it imparts a sour taste affecting the flavor of the fermented product. It has been reported that the flavor of sourdough products depends on the ratio of acetic acid to lactic acid [4].

Water-Soluble Free Sugar Content of Rice Sourdoughs after Fermentation
The sugar content of rice sourdoughs fermented with the five different makgeolli was measured by HPLC (Fig. 3). After 6.5 h of fermentation, the sucrose content decreased in all samples, while the glucose and fructose contents increased. In particular, samples a–d showed complete depletion of sucrose after fermentation. This can be attributed...
Distribution of Different LAB in Rice Sourdoughs

The distribution of LAB in the different rice sourdough samples was analyzed morphologically based on the hydrolytic activities of microbial enzymes present in the sourdough [14].

**Table 1.** Lactic acid bacteria (LAB) isolated from five different fermented rice doughs (a–e) using modified MRS and SDB media.

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Isolated LAB</th>
<th>Fermented rice doughs&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Lactobacillus plantarum</em></td>
<td>a  5  b  8  c  1</td>
</tr>
<tr>
<td>2</td>
<td><em>Lactobacillus brevis</em></td>
<td>a  2  b  3  c  3</td>
</tr>
<tr>
<td>3</td>
<td><em>Lactobacillus casei</em></td>
<td>1  2  4</td>
</tr>
<tr>
<td>4</td>
<td><em>Lactobacillus paracasei</em></td>
<td>a  2  b  1  c  3</td>
</tr>
<tr>
<td>5</td>
<td><em>Lactobacillus crустorum</em></td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td><em>Lactobacillus fermentum</em></td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td><em>Lactobacillus harbinensis</em></td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td><em>Lactobacillus zae</em></td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td><em>Lactobacillus pentosus</em></td>
<td>a  1  b  1  c  1</td>
</tr>
<tr>
<td>10</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td><em>Leuconostoc citreum</em></td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td><em>Leuconostoc pseudomesenteroides</em></td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>*</sup>Numbers in the above table indicate the number of isolates from fermented rice dough.

Fig. 2. Acetic acid (-) and lactic acid (—) produced during fermentation (6.0 h) of rice dough samples made with five different makgeolli (a–e).

Fig. 3. Water-soluble free sugar contents of rice doughs after 6.5 h of fermentation.
method of Lee and Lee [15]. A total of 52 LAB strains were isolated from the jeung-pyun doughs as pure cultures using selective media (BPB-MRS or BPB-SDB). The isolates belonged to the genera *Lactobacillus* and *Leuconostoc* (Table 1). *Lactobacillus plantarum* (15 isolates), isolated from samples a–d, was the predominant species, accounting for about 30% of the isolates. *Lb. brevis* (8 isolates), isolated from samples a–d, was the next most common species, accounting for about 16% of the isolates. *Lb. casei* (7 isolates) and *Lb. paracasei* (6 isolates) accounted for about 14% and 12% of the isolates from the five samples, respectively. In addition *Lb. pentosus* (3 isolates), *Lb. rhamnosus* (2 isolates), *Lb. crustorum* (1 isolate), *Lb. fermentum* (1 isolate), *Lb. harbinensis* (1 isolate), *Le. zeae* (1 isolate), *Leuconostoc citreum* (1 isolate), *Le. mesenteroides* (1 isolate), and *Le. pseu demonstrated diverse LAB profiles in rice doughs fermented with different commercial makgeolli.

### Microbial Diversity Analysis by PCR-DGGE and 16S/26S rRNA Gene Sequencing

PCR-DGGE analysis followed by 16S/26S rRNA gene sequencing was performed at various time points during the fermentation period to determine the biodiversity of LAB and yeast in rice sourdoughs. The PCR-DGGE gels of the LAB strains are shown in Fig. 4A and the results of the sequence analysis are presented in Table 2. The gene sequences exhibited more than 98% identity with sequences in the GenBank database. All of the jeung-pyun doughs fermented with makgeolli (a to e) displayed 4 to 5 bands in different positions that corresponded to various LAB. According to the 16S rRNA gene sequence analysis, most of the bands (2, 3, 5, 6, and 11–16) corresponded to *Lactobacillus* species. The species were identified as *Lactobacillus* spp. (bands 2 and 15), *Lb. plantarum* (band 3), *Lb. brevis* (band 5), *Lb. crustorum* (band 6), *Lb. harbinensis* (bands 11 and 14), and *Lb. pentosus* (bands 13 and 16). Band 1 belonged to *Weissella*.
Microbial Analysis of Korean Fermented Rice Dough

Confusa and band 4 was assigned to W. paramesenteroides. Bands 7–9 and 18, which were intense in all five fermented dough samples, corresponded to rice genes (Oryza sativa Indica Group). The above results showed the growth of various LAB in rice doughs depending on different commercial makgeolli used. Some of the common LAB observed in fermented rice doughs are Lb. plantarum, Lb. pentosus, and Lb. brevis. The PCR-DGGE analysis of yeast (Fig. 4B) showed two major bands in all samples (a to e), regardless of fermentation time or makgeolli used, which indicated that only one type of yeast was used as a starter in makgeolli fermentation. The yeast was identified as Saccharomyces cerevisiae and showed 100% identity with S. cerevisiae L610 and S. cerevisiae LE013 (Table 2).

### Discussion

Current research on sourdough fermentation is focused on the identification of microflora and investigation of the carbohydrates being metabolized by the LAB and yeast species. Fermentation not only improves the texture and flavor of dough but also protects the dough from spoilage by molds and bacteria. Lactobacillus species are the predominant LAB involved in wheat sourdough fermentation. Some are facultative or obligatory heterofermentative lactobacilli, such as Lb. sanfranciscensis, Lb. plantarum, Lb. alimentarius, Lb. pontis, Lb. brevis, and Lb. reuteri [16, 17]. In the present study, a polyphasic microbial analysis of rice sourdough fermented with commercial makgeolli was performed. Our culture-dependent and culture-independent analyses showed that various Lactobacillus species (e.g., Lb. plantarum, Lb. pentosus, and Lb. brevis), and S. cerevisiae were predominant in the rice sourdough. It has been reported that Lb. sanfranciscensis is the predominant LAB involved in fermentation of sourdough [18, 19]. Generally, the growth of Lb. sanfranciscensis depends on the availability of specific amino acids and peptides present in sourdough [16, 20], since Lb. sanfranciscensis cannot synthesize most of its required cofactors and vitamins. Moreover, the absence of extracellular protease (prt) genes in the genome of Lb. sanfranciscensis reflects its high adaptation to and associated dependency on wheat-based sourdough [6, 11]. However, Lb. sanfranciscensis was not detected in rice dough fermented with makgeolli. This could be due to exposure to the alcohol (6.0–8.0%) in makgeolli [4], since Ganzle et al. [22] reported that Lb. sanfranciscensis cannot tolerate an alcohol content beyond 5.0%, or it may be due to the absence of cofactors, vitamins, peptides, or amino acids in rice sourdough that are required for the growth and proliferation of Lb. sanfranciscensis [6, 21].

Generally, the quality of any fermented product depends on the type of microorganism involved in the fermentation. In the present study, rice doughs fermented with five different commercial makgeolli showed significant differences in final pH, organic acid production, viable cell count, and reducing sugar content. These results imply that jeungpyun prepared using different makgeolli have different tastes and flavors. Hence, there is a need to optimize the LAB starter for the production of good-quality rice cake. Among the five makgeolli samples tested, makgeolli b showed unique results in terms of sugar utilization, growth of microorganisms, and production of organic acids. Heterofermentative LAB produce lactic acid, acetic acid, and carbon dioxide as byproducts. The organic acids

<table>
<thead>
<tr>
<th>Samples</th>
<th>Band no.</th>
<th>Species</th>
<th>Identity</th>
<th>Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>1</td>
<td>Weissella confusa</td>
<td>98%</td>
<td>KC568546.1</td>
</tr>
<tr>
<td>LAB</td>
<td>3, 12</td>
<td>Lactobacillus plantarum</td>
<td>100%</td>
<td>KC352738.1</td>
</tr>
<tr>
<td>LAB</td>
<td>4</td>
<td>Weissella paramesenteroides</td>
<td>99%</td>
<td>AB775181.1</td>
</tr>
<tr>
<td>LAB</td>
<td>5</td>
<td>Lactobacillus brevis</td>
<td>100%</td>
<td>KC755092.1</td>
</tr>
<tr>
<td>LAB</td>
<td>6</td>
<td>Lactobacillus crustorum</td>
<td>100%</td>
<td>KC755094.1</td>
</tr>
<tr>
<td>Rice</td>
<td>7, 8, 9, 18</td>
<td>Oryza sativa Indica Group</td>
<td>100%</td>
<td>JN861110.1</td>
</tr>
<tr>
<td>LAB</td>
<td>11, 14</td>
<td>Lactobacillus harbinensis</td>
<td>100%</td>
<td>AB368916.1</td>
</tr>
<tr>
<td>LAB</td>
<td>2, 15</td>
<td>Lactobacillus sp.</td>
<td>99%</td>
<td>JX826539.1</td>
</tr>
<tr>
<td>LAB</td>
<td>13, 16</td>
<td>Lactobacillus pentosus</td>
<td>99%</td>
<td>HP936872.1</td>
</tr>
<tr>
<td>LAB</td>
<td>17</td>
<td>Leuconostoc pseudomesenteroides</td>
<td>100%</td>
<td>AF515228.1</td>
</tr>
<tr>
<td>Yeast</td>
<td>1</td>
<td>Saccharomyces cerevisiae L610</td>
<td>100%</td>
<td>JQ518268.1</td>
</tr>
<tr>
<td>Yeast</td>
<td>2</td>
<td>Saccharomyces cerevisiae LE013</td>
<td>100%</td>
<td>JQ518268.1</td>
</tr>
</tbody>
</table>
produced by heterofermentative LAB improve the flavor and shelf life of fermented products and inhibit the growth of pathogenic molds and bacteria [23].

The sugars present in the rice dough fermented with makgeolli could be consumed by facultative heterofermentative Lactobacillus species to produce dextran and oligosaccharides in the presence of dextranucrase. Dextran is a microbial exopolysaccharide that improves bread volume and firmness and preserves freshness during storage [24]. Maltose present in rice dough can act as an acceptor molecule in the presence of sucrose, resulting in the formation of oligosaccharides. Moreover, the dextran and the oligosaccharides produced during fermentation are not digested by yeast, leading to enhanced quality and a high content of putative prebiotic oligosaccharides in jeung-pyun [25, 26]. The glucose and fructose released during this fermentation process are consumed by different LAB strains and yeast in the sourdough to produce organic acids, carbon dioxide, and alcohol, which further enhance the quality of the product. The organic acids released during sourdough fermentation decrease the pH of the sourdough and inhibit the activity of endoamylase, preventing the hydrolysis of starch during baking and enhancing the quality of bread [20].

In conclusion, the LAB and yeast microbiota in rice sourdoughs fermented after the addition of five different makgeolli were identified by both culture-dependent and culture-independent methods. It was observed that rice doughs fermented with different makgeolli showed different pH (4.12–4.79), total titratable acidity (0.54–0.81%), and growth profiles (5.2 to 8.2 Log CFU/g). The 16S rRNA gene sequencing and PCR-DGGE analyses showed the wide distribution of LAB in the fermented rice dough samples. The predominant species were Lb. plantarum, Lb. pentosus, and Lb. brevis, and only one yeast strain belonging to S. cerevisiae. From the above studies, we confirm that makgeolli samples are not recommended as starters for the standardized production of rice dough fermentation because of their varied LAB profiles. In order to produce a high-quality jeung-pyun at industrial scale, we need to develop LAB starters with desired quality (pH, organic acid, and exopolysaccharides production) to enrich the flavor and shelf-life of the products.

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References


