Identification of LAB and Fungi in Laru, a Fermentation Starter, by PCR-DGGE, SDS-PAGE, and MALDI-TOF MS

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Introduction

Laru is a dry, dusty, flattened starter that is used to prepare a fermented rice dish called tapai. Laru is known by various names according to the region in which it is made: laru (Brunei Darussalam and Sabah) [1], ragi tapai (Malaysia) [2], ragi tapé (Indonesia) [3, 4], and look-pang (Thailand) [5, 6]. Traditionally, laru is prepared by mixing rice flour with dry, ground spices such as garlic (Allium sativum), galangal root (Alpinia galanga), black pepper (Piper nigrum), cane sugar (Saccharum officinarum), lemon (Citrus aurantiacum var. fusca), and coconut water (Cocos nucifera) [7], before being inoculated with dry powdered ragi from previous batches [8].

The majority of the starters are small (3–6 cm), round, flattened cakes of rice flour that are air- or sun-dried. Since tapai starters are mainly manufactured aseptically by villagers, different combinations of ingredients are used and are typically not disclosed [1]. The microbiota composition also differs according to the combination of ingredients and the country from which the ingredients were obtained. According to Atmodjo [9], a good ragi must be able to inhibit the growth of undesirable microbes.

Saccharomyces cerevisiae, Rhizopus oryzae, and Endomycopsis fibuliger have previously been detected in tapai starters [3, 8, 10, 11]. Moreover, Weissella spp., Enterococcus spp., and Pediococcus pentosaceus, the three main Lactic acid bacteria (LAB) in ragi tapé, have been consistently detected throughout tapai fermentation [12]. LAB are believed to be particularly important toward the end of tapai fermentation by contributing compounds that enhance the flavor and/or by killing undesirable microorganisms [13]. Hesseltine and Ray [13] analyzed samples from Bali, Java, and Nepal and found that Pediococcus was the predominant genus in tapai starters, regardless of the country of origin. In another study, Sujaya et al. [12, 14] detected P. pentosaceus in nine ragi tapé samples obtained from different parts of Indonesia.

Both culture-independent (polymerase chain reaction-denaturant gradient gel electrophoresis (PCR-DGGE)) and culture-dependent methods (sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption ionization time-of-flight mass
spectrometry (MALDI-TOF MS)) have been used to detect LAB and fungi in laru samples. PCR-DGGE, which is able to detect both live and dead unculturible cells, enables the rapid analysis of species and changes in microbial communities [15, 16]. In contrast, SDS-PAGE analysis of whole-cell protein extracts is an easy and quick method to identify large numbers of strains with sufficient taxonomic resolution at both the species and subspecies levels if conducted under highly standardized conditions [17]. MALDI-TOF MS, a chemotaxonomic method in which LAB species are identified on the basis of mass spectral patterns of ribosomal proteins, can also be used to confirm 16S rRNA and 18S rRNA gene sequencing results derived from SDS-PAGE protein band groupings [18–20].

We aimed to implement both culture-independent and culture-dependent methods (PCR-DGGE, SDS-PAGE, and MALDI-TOF MS) for the identification of LAB and fungal communities from randomly selected laru samples collected from the upper parts of Borneo Island (Brunei Darussalam, Sabah, and Sarawak). Since the laru samples analyzed in this study were prepared traditionally, we predicted a high diversity of bacteria and fungi from the natural environments. The data obtained from this study will be useful for understanding the microbiological content of traditionally prepared laru for tapai fermentation and for determining how differences in microbiological content account for differences in time until consumption, quality, shelf life, alcohol content, and sugar content in tapai products. Thus, our results will also be useful for the development of refined starters for uniform tapai production.

Materials and Methods

Isolation of LAB and Fungi from Laru Samples

Seventeen laru samples were purchased from randomly chosen markets along the upper coast of Borneo Island in 2015. The samples were manufactured in the following regional groups: region K (samples 2, 6, and 11: Kota Belud, Sabah, Malaysia), region L (sample 8: Lawas, Sarawak, Malaysia), region E (sample 10: Beaufort, Sabah, Malaysia), region T (samples 12 and 15: Tawau, Sabah, Malaysia), region P (sample 1: Penampang, Sabah, Malaysia), region D (sample 4: Donggongon, Sabah, Malaysia), and region B (samples 3, 5, 7, 9, 13, 14, 16, and 17: Labi, Belait, Brunei).

Ten grams of each homogenized sample was aseptically weighed and transferred to a sterile stomacher filter bag (BA6141/STR; Seward, UK). Next, 90 ml of sterile water was added, and the suspension was mixed in a stomacher apparatus (Circular Stomacher 400; Seward, USA) for 60 sec. Appropriate serial dilutions (10^−10) were plated in duplicate on Man, Rogosa, and Sharpe agar (MRS) (Difco, USA) and incubated at 30°C for 48 h under anaerobic conditions using an Anaeropack instrument (Mitsubishi Gas Chemical, Japan). Colonies were also grown on yeast extract-glucose-chloramphenicol agar (YGC) (MBcell, Korea) at 30°C for 48 h under aerobic conditions. LAB and fungal colonies were randomly subcultured in MRS and Sabouraud broth (MBcell), respectively. Each isolate was mixed with 80% (v/v) glycerol at a 7:3 (isolate:glycerol) ratio and stored at −80°C for further use.

DNA Extraction

Each homogenized sample was filtered through two layers of cheesecloth prior to DNA extraction. Filtrates were centrifuged at 16,200 x g for 15 min at 4°C to obtain cell pellets, which were then washed with sterile water. DNA was then extracted from the pellets using a commercial genomic DNA preparation kit (MB113, Bacterial Genomic DNA Extraction Kit; BioSolution, Korea) according to the manufacturer’s instructions. The yield and quality of the DNA were visualized after electrophoresis on a 1% agarose gel.

PCR-DGGE Analysis

The PCR products were analyzed on a 2% agarose gel before DGGE analysis. PCR-DGGE analysis was conducted according to the protocols described by Kim et al. [17]. Briefly, the 16S rRNA and 18S rRNA genes were amplified from microbial community DNA using the 27F/1492R 16S universal primers and NS1/FRI primers (Bionics, Korea), respectively, under the following thermocycling conditions: 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min. These cycles were followed by extension at 72°C for 10 min, followed by cooling to 4°C. The V3 region of the 16S rRNA gene and the 18S rRNA genes were reamplified from the PCR products using the DGGE primers GC-338F/518R and NS3-GC/YM951R, respectively, under the following thermocycling conditions: 95°C for 5 min, 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and finally 5 min at 72°C, followed by cooling to 4°C. The sequences of the DGGE forward (GC-338F) and reverse (518R) primers are provided in Table 1.

The PCR amplifications were performed in a Mastercycler instrument (Eppendorf, Germany) in a final volume of 25 μl consisting of 5 μl of template, 2.5 μl of 10× PCR buffer, 2 μl of dNTP mixture (2.5 mM each), 0.1 μl of Taq polymerase (5 U μl−1; Takara Biotechnology, Japan), and 0.4 μM of each primer. The PCR products were analyzed on a 2% agarose gel before DGGE analysis.

The resultant amplicons were mixed with 5 μl of 6× loading dye and directly loaded onto 80 g/l polyacrylamide gels with a denaturing gradient of 20% to 50% urea-formamide. The gels were processed in 1× TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0) on a Dcode Universal Mutation Detection system (Bio-Rad, USA) for 30 min at 40 V and 15.5 h at 60 V. The gels were then stained with ethidium bromide for 30 min, after which images were captured using a Quantum ST4 1100 system (ST4V16.07; Vilber Lourmat, France).

Sterile blades were used to excise bands of interest from the gels. The gel slices were incubated overnight at 4°C in ultra-