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

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Samples of *Laru* (a fermentation starter) obtained from the upper part of Borneo Island were analyzed for their lactic acid bacteria (LAB) and fungal diversity using both a culture-independent method (PCR-DGGE) and culture-dependent methods (SDS-PAGE and MALDI-TOF MS). *Pediococcus pentosaceus, Lactobacillus brevis, Saccharomycopsis fibuligera, Hyphopichia burtonii*, and *Kodamaea ohmeri* were detected by all three methods. In addition, *Weissella cibaria, Weissella paramesenteroides, Leuconostoc citreum, Leuconostoc mesenteroides, Lactococcus lactis, Rhizopus oryzae/Amylomyces rouxii, Mucor indicus*, and *Candida intermedia* were detected by PCR-DGGE. In contrast, *Lactobacillus fermentum, Lactobacillus plantarum, Pichia anomala, Candida parapsilosis*, and *Candida orthopsilosis* were detected only by the culture-dependent methods. Our results indicate that the culture-independent method can be used to determine whether multiple laru samples originated from the same manufacturing region; however, using the culture-independent and the two culture-dependent approaches in combination provides a more comprehensive overview of the laru microbiota.

Keywords: Laru, ragi, lactic acid bacteria, PCR-DGGE, SDS-PAGE

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 galangal*), 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 unculturable 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 ($10^1 - 10^8$) 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 ×*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/FR1 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 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 µ/l; Takara Biotechnology, Japan), and 0.4 pM 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-

Table 1. Sequences of the primers used in this study

Target	Primer		Sequence (5′ → 3′)	Reference
Bacteria	First PCR	27F (F)	AGAGTTTGATCCTGGCTCAG	[21]
		1492 (R)	GGCTACCTTGTTACGACTT	
	Nested PCR	GC-338f (F)	CGCCCGCCGCGCGGCGGGCGGGGCGGGGGACGGGGGACTCCTACGGGAGGCAGCAG	[22]
		518r (R)	ATTACCGCGGCTGCTGG	
Fungi	First PCR	NS1 (F)	GTAGTCATATGCTTGTCTC	[23]
		FR1 (R)	AICCATTCAATCGGTAIT	
	Nested PCR	NS3 (F)	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGCAAGTCTGGTGC-CAGCAGCC	[24]
		YM951r (R)	TTGGCAAATGCTTTCGC	

filtered water to allow passive diffusion of the DNA from the polyacrylamide matrix into the water for use as template for re-amplification using the DGGE primers GC-338F/518R and GC-NS3/YM951R. The resultant PCR products were run again on polyacrylamide gels to improve the band purity for sequencing, after which the excised gel slices were incubated overnight at 4°C.

Sequencing of DGGE Bands

The eluted DNA was amplified using the same primer pairs, but without the GC clamp. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, USA). Sequences were determined using an automated DNA sequencer (Jenotech, Korea). Partial ribosomal DNA sequences from the laru samples were searched against the GenBank database using BLAST [25] to identify the closest known relatives.

SDS-PAGE Whole-Cell Protein Extract Analysis and 16S rRNA/18S rRNA Gene Sequencing

Proteins in whole-cell extracts from strains cultured in MRS and YGC media were denatured before SDS-PAGE was performed according to the protocols described by Jung *et al*. [26]. The SDS-PAGE gels were scanned with a high-resolution Perfection V700 Photo Scanner (Epson, USA), and whole-cell patterns were grouped according to protein band pattern. Chromosomal DNA of selected LAB isolates from each group was extracted, and the 16S rRNA gene was amplified by PCR using 27F as a forward primer and 1492R as a reverse primer (Bionics) [26]. The 18S rRNA gene of the fungal isolates was amplified using a forward primer (NS1) and reverse primer (FR1). The primer sequences are provided in Table 1. The purified PCR products were sequenced using an automated DNA sequencer (Jenotech, Korea). An NCBI BLAST homology search (BLASTN) [25] using the obtained sequences was used to identify the LAB and fungi present in the laru samples.

Identification of LAB and Fungi Using MALDI-TOF MS

To identify LAB isolates, a single colony isolated on MRS medium (30°C, 24 h) was spotted on a MSP 96-target polished steel plate (Bruker Daltonik GmbH, Germany). Each colony spot was overlaid with 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid (g/l) in acetonitrile:water:trifluoracetic acid (50:47.5:2.5)). The matrix-sample mixture was crystallized by air-drying at room temperature for 5 min, loaded in a mass spectrometer, and subjected to MALDI-TOF MS analysis.

Identification of fungal isolates was performed as described by Pavlovic *et al.* [27]. Briefly, isolated colonies of fungal isolates grown on YGC agar (30°C, 48 h) were picked and suspended in 300 µl of sterile water. After thorough mixing, 900 µl of absolute ethanol was added before centrifugation at 9,600 ×*g* for 2 min. The resultant supernatant was discarded, the pellet was centrifuged again, and the residual ethanol was removed completely before drying at room temperature. After drying the pellet, 30 µl of formic acid (70% (w/v)) was added, and the mixture was vortexed. Next, 30 µl of acetonitrile (80% (w/v)) was added, followed by vortexing. The solution was centrifuged at maximum speed for 2 min, after which 1.5 µl of the supernatant was spotted onto a polished MALDI target plate (Bruker Daltonik), allowed to air-dry, and then overlaid with 1.5 µl of matrix solution. The matrix solution consisted of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany) in 50% (w/v) acetonitrile and 0.25 g/l trifluoroacetic acid.

A Microflex LT Biotyper MALDI-TOF MS system (Bruker Daltonik) equipped with a 337 nm nitrogen cartridge laser (MNL106; Bruker Daltonik) was used for all analyses. The Biotyper instrument system was controlled using Flexcontrol ver. 3.4 software. Ions were accelerated in a 20 kV electric field through a grid and separated according to mass to charge (*m/z*) ratio in a 95-cm-long linear field free drift region. External calibration was performed prior to the analyses with the Bruker Bacterial Test Standard. For each isolate, all spectra generated in the mass range of 2–20 kDa were automatically acquired and imported into Biotyper ver. 3.1. The mass spectra were analyzed by standard pattern matching against the spectra of the 5,627 species in the MALDI Biotyper database (DB4613; Bruker Daltonik). Identification was considered complete when the spots for a given strain yielded a score ≥1.7.

Results and Discussion

PCR-DGGE Analysis

We used PCR-DGGE, a culture-independent method that

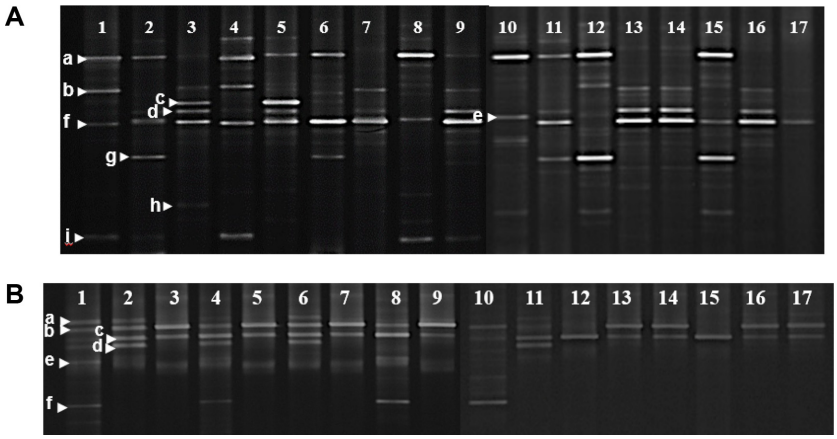

Fig. 1. DGGE patterns of 16S V3 rRNA gene sequences (**A**) and 18S rRNA gene sequences (**B**) in the laru samples (1–17).

utilizes nested PCR products, to detect the presence of LAB and fungi in the laru samples. DGGE images were used to compare bacterial (Fig. 1A) and fungal (Fig. 1B) communities in the 17 laru samples. The corresponding sequencing results are listed in Table 2.

The identified LAB included *Weissella*, *Lactobacillus*, and *Pediococcus* spp., all of which have been previously reported [12, 28], as well as *Lactococcus* sp. The identified fungi included *Saccharomycopsis*, *Hyphopichia*, *Rhizopus/Amylomyces*, *Mucor*, and *Candida* spp., all of which have also been documented [3, 7], and *Kodamaea* sp. Overall, PCR-DGGE identified the following LAB: two *Weissella* species (*W. cibaria* and *W. paramesenteroides*), one *Pediococcus* species (*P. pentosaceus*), one *Lactobacillus* species (*L. brevis*), two *Leuconostoc* species (*Leu. citreum* and *Leu. mesenteroides*), and one *Lactococcus* species (*Lc. lactis*). With respect to fungi, PCR-DGGE identified one *Saccharomycopsis* species (*Sc. fibuligera*), one *Hyphopichia* species (*H. burtonii*), one *Rhizopus/Amylomyces* species (*R. oryzae/ A. rouxii*), one *Mucor* species (*M. indicus*), one *Kodamaea* species (*K. ohmeri*), and one *Candida* species (*C. intermedia*).

The most predominant LAB was *L. brevis* (band f in Fig. 1A), which was present in all samples except 10 and 12. *W. cibaria* (band a in Fig. 1A) was present in all samples except 3, 7, 9, 13, 14, 16, and 17, whereas *Leu. citreum* (band b and Fig. 1A) was not present in samples 2, 5, 6, 8, 10, 11, 15, or 17. *Lc. lactis* (band i in Fig. 1A) was only detected in samples 1, 2, 4, 8, and 9. The presence of *Lc. lactis* has not been reported by the studies conducted from Sujaya *et al.* [12, 28] and Ardhana and Fleet [29] for microbial diversity in ragi tapé fermentation. *Leu. mesenteroides* (band c in Fig. 1A) was detected in samples 3, 5, 23, 24, and 16. Only sample 10 was found to contain *W. paramesenteroides* (band e in Fig. 1A). In contrast to the studies conducted by Hesseltine and Ray [13] and Sujaya *et al.* [12, 14], in which *P. pentosaceus* was reported to be the predominant LAB, *P. pentosaceus* (band g in Fig. 1A) was detected only in samples 2, 6, 11, 12, and 15.

R. oryzae/A. rouxii (band c in Fig. 1B) was the predominant fungus and was detected in all laru samples. *H. burtonii* (band b in Fig. 1B) was present in all samples except 12 and 15. *Sc. fibuligera* (band a in Fig. 1B) and *M. indicus* (band d

Table 2. Identification of bacterial and fungal species in the laru samples by sequencing the 16S V3 rRNA and 18S rRNA fragments excised from PCR-DGGE gels

DGGE	Band no.	Species identification	Homology (%)	Accession No.
Bacteria	a	*Weissella cibaria*	100	JN851745.1
	b	*Leuconostoc citreum*	97	LC096222.1
	c	*Leuconostoc mesenteroides*	99	JN863609.1
	d	*Staphylococcus haemolyticus*	99	KT026096.1
	e	*Weissella paramesenteroides*	99	KP189212.1
	f	*Lactobacillus brevis*	99	JN863616.1
	g	*Pediococcus pentosaceus*	100	JN851781.1
	h	*Staphylococcus kloosii*	99	KF233801.1
	i	*Lactococcus lactis*	99	EF204360.1
Fungi	a	*Saccharomycopsis fibuligera*	100	KP119822.1
	b	*Hyphopichia burtonii*	99	JQ698903.1
	c	*Rhizopus oryzae/ Amylomyces rouxii*	100	KJ408539.1/ KJ588788.1
	d	*Mucor indicus*	99	KM527229.1
	e	*Kodamaea ohmeri*	99	KM006493.1
	f	*Candida intermedia*	100	EF408189.1

in Fig. 1B) were present in samples 1, 2, 4, 6, and 11. *C. intermedia* (band f in Fig. 1B) was present in samples 1, 4, 8, and 10, whereas *K. ohmeri* (band e in Fig. 1B) was only present in sample 1. *K. ohmeri*, previously known as *Pichia ohmeri* and *Yamadazyma ohmeri*, is commonly used in the food industry for its ability to prevent fruit spoilage [30]. However, *K. ohmeri* has been reported to be an emerging opportunistic fungal pathogen because it can infect immunocompromised patients [31]. The presence of these fungi indicates that all 17 of the laru samples are capable of producing good quality tapai, since tapai is dependent on the presence of at least one amylolytic filamentous fungus and at least one alcohol-producing yeast [32]. Although Ellis *et al.* [33] concluded that the presence of the fungus *A. rouxii* was essential for the production of tapai, *A. rouxii* cannot be discriminated from the lactic acid-accumulating group of *R. oryzae* owing to their identical amplified sequences [34].

The LAB and fungal profiles displayed by the PCR-DGGE bands (Figs. 1A and 1B, respectively) were next used to determine whether or not the samples originated from the same source or region. Samples 2, 6, and 11 originated from region K and shared *W. cibaria*, *L. brevis*, *P. pentosaceus*, *Sc. fibuligera*, *H. burtonii*, *R. oryzae/A. rouxii*, and *M. indicus* in common; however, sample 2 also contained *Lc. lactis*. Manufactured in region T, both samples 12 and 15 contained *W. cibaria*, *P. pentosaceus*, and *R. oryzae/A. rouxii*; *Leu. citreum* was also found in sample 12, and *L. brevis* was found in sample 15. Although samples 5, 7, 9, 13, 14, 16, and 17 were manufactured by a single company in region B, the overall microbial compositions were somewhat discrepant. However, these samples did share *L. brevis*, *H. burtonii,* and *R. oryzae/A. rouxii* in common, and *Leu. citreum* and *Leu. mesenteroides* were detected in almost all samples. This finding suggests that the production of laru samples by this company was inconsistent, and that the laru had been manufactured under poorly controlled conditions. Samples 8 and 10 were purchased and manufactured in regions L and E, respectively. These samples shared *W. cibaria*, *L. brevis*, *H. burtonii*/*A. rouxii*, and *C. intermedia* as common microorganisms, whereas sample 8 had *Lc. lactis*, and sample 10 had *W. paramesenteroides*. Although samples 1 and 4 originated from regions P and D, respectively, these samples were used to prepare alcoholic beverages that underwent 3 weeks of fermentation instead of 24 to 48 h. This finding explains why these samples had the most diverse LAB and fungi, specifically *W. cibaria*, *Leu. citreum*, *L. brevis*, *Lc. lactis*, *S. fibuligera*, *H. burtonii*, *R. oryzae/A. rouxii*, *M. indicus*, and *C. intermedia*; additionally, sample 1 was found to have *K. ohmeri*.

SDS-PAGE Whole-Cell Protein Extract Analysis and 16S rRNA/18S rRNA Gene Sequencing

The patterns resulting from SDS-PAGE analysis of whole-cell protein extracts were compared and classified into four groups (1-4, 2-10, 1-7, and 1-9) of LAB strains and seven groups (4-6y, 6-2y, 1-1y, 9-7y, 4-12y, 1-7y, and 4-3y) of fungus strains (Fig. 2). Isolates were further analyzed by 16S rRNA and 18S rRNA gene sequencing. The identities of the LAB isolates and fungal isolates from the SDS-PAGE patterns and their corresponding NCBI accession numbers are listed in Table 3. Among the four LAB groups, *L. fermentum* (1 isolate, 10%), *L. plantarum* (3 isolates, 30%), and *L. brevis* (1 isolates, 10%) were only found in sample 1 and *P. pentosaceus* (5 isolates, 50%) was detected in samples 2 and 15. The identification and distributions of the seven fungal groups are shown in Table 3. *P. anomala* (88 isolates, 68.2%) was the most predominant fungus and was detected in all samples except 12. *P. anomala* was consistently isolated from samples 3, 5, 7, 9, 13, 14, 16, and 17, which were manufactured from region B; however, the number of isolates from each sample varied widely (range, 3–13 isolates). Samples 2, 6, and 11 from region K also contained *P. anomala* (1, 1, and 12 isolates, respectively), whereas this fungus was only isolated from one of the samples originating from region T (8 isolates). This finding indicates that it would be very difficult to predict if these samples had been manufactured by the same company or had originated from the same region if only data from the culture-dependent method were available. The next most predominant fungus was *Sc. fibuligera* (17 isolates, 13.2%), which was detected in samples 2, 6, 7, 8, 9, 11, 13, 15, and 16. *Sc. fibuligera* was successfully isolated from five of the eight samples that were manufactured in region B and two of the three samples originating from region K. *H. burtonii* (16 isolates, 12.4%) was found in samples 2, 3, 5, 7, 9, 11, 13, 14, and 17. Other fungi such as *C. parapsilosis* (4 isolates, 3.1%) and *S. cerevisiae* (2 isolates, 1.6%) were also detected in sample 1, whereas *K. ohmeri* (1 isolate, 0.78%) and *C. orthopsilosis* (1 isolate, 0.78%) were identified in sample 4. These results indicate that fungi are the major culturable constituent of laru using the culture conditions selected, and that *P. anomala, Sc. fibuligera,* and *H. burtonii* are the most widespread fungal species. Since sample 1 was used to ferment alcoholic beverages, it contained the most diverse culturable fungi using this method. The microorganisms

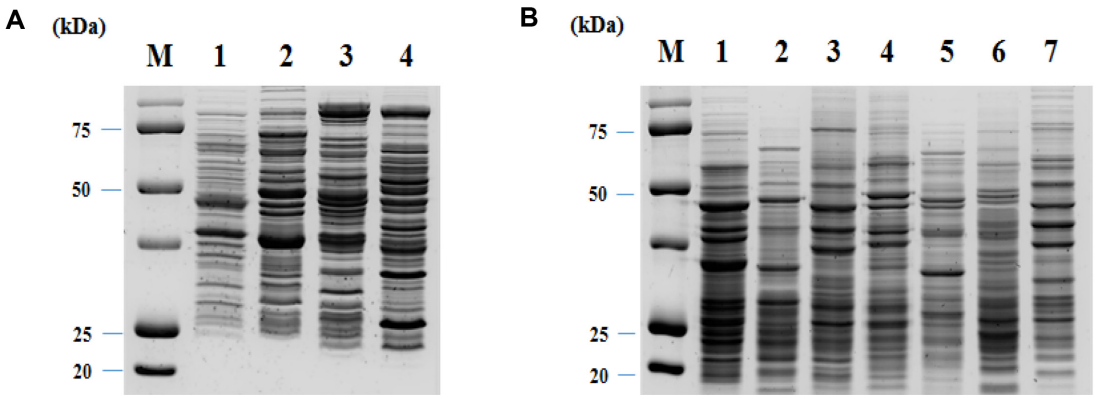

Fig. 2. SDS-PAGE whole-cell protein patterns of lactic acid bacteria (**A**) and fungi (**B**) representative of the four LAB and seven fungi groups isolated from laru samples.

(**A**) Lanes: M, protein Mw markers (kDa); 1, 1-4; 2, 2-10; 3, 1-7; 4, 1-9. (**B**) Lanes: M, protein Mw markers (kDa); 1, 4-6y; 2, 6-2y; 3, 1-1y; 4, 9-7y; 5, 4-12y; 6, 1-7y; 7, 4-3y.

Table 3. Identification of LAB and fungal isolates selected from SDS-PAGE patterns of whole-cell protein extracts by 16S rRNA and 18S rRNA gene sequencing analyses.

	Isolate no. of SDS-PAGE pattern[a]	Species identification (NCBI Accession No.)	Homology[c] (%)
LAB	1-7[b]	*Lactobacillus fermentum* (JF903803.1)	99
	1-4	*Lactobacillus plantarum* (KR336551.1)	100
	2-10	*Pediococcus pentosaceus* (KJ477378.1)	100
	1-9	*Lactobacillus brevis* (AB969780.1)	99
Fungi	1-7y	*Saccharomyces cerevisiae* (GQ458028.1)	99
	4-6y	*Pichia anomala* (EF427893.1)	99
	6-2y	*Saccharomycopsis fibuligera* (KP119820.1)	99
	9-7y	*Hyphopichia burtonii* (JQ698903.1)	99
	1-1y	*Candida parapsilosis* (KT229545.1)	99
	4-12y	*Kodamaea ohmeri* (HQ412607.1)	99
	4-3y	*Candida orthopsilosis* (AY 520277.1)	99

[a]Isolate No. designation refers to that in Fig. 2.

[b]Laru samples 1–17.

[c]16S/18S rRNA gene sequences of the LAB and fungal strains were searched against the NCBI sequence database.

involved in the fermentation of sample 1 were studied by Chiang *et al.* [35]. However, sample 4, which was cultured for a similar purpose, showed different results.

Identification of LAB and Fungal Isolates Using MALDI-TOF MS

In MALDI-TOF MS analysis, ribosomal proteins produce a fingerprint in the form of a mass spectrum. We selected a number of the identified isolates for MALDI-TOF MS analysis. The LAB and fungi results obtained from MALDI-TOF MS analysis were consistent with those based on whole-cell protein extract patterns and 16S/18S rRNA gene sequence analyses (Table 4), except *Sc. fibuligera*, which was not available in the MALDI-TOF MS database. Since the results from SDS-PAGE whole-cell protein extract analysis and 16S rRNA/18S rRNA gene sequencing were identical to those from MALDI-TOF MS, either SDS-PAGE or rRNA gene analysis is an appropriate culture-dependent approach. The most appropriate technique should be chosen on the basis of the duration of analysis, cost of equipment, space for equipment, and availability of data library.

In general, the culture-independent method showed that all 17 laru samples contained at least one LAB species (Fig. 1A), whereas the culture-dependent methods detected LAB in only three samples (Table 3). The LAB species detected by the culture-independent method were

Table 4. Comparison of the 16S and 18S rRNA gene sequencing and MALDI-TOF MS results.

rRNA gene sequencing		MALDI-TOF MS (Score value)
16S	*Lactobacillus fermentum*	*L. fermentum* DSM 20391 DSM (1.864)
	Lactobacillus plantarum	*L. plantarum* DSM 12028 DSM (2.045)
	Pediococcus pentosaceus	*P. pentosaceus* DSM 20206 DSM (1.899)
	Lactobacillus brevis	*L. brevis* DSM 20054T DSM (1.951)
18S	*Saccharomyces cerevisiae*	*S. cerevisiae* isolate LGL Muenchen (1.817)
	Pichia anomala	*P. anomala* DSM 70260 (2.079)
	Hyphopichia burtonii	*H. burtonii* MY00872_06 ERL (1.764)
	Candida parapsilosis	*C. parapsilosis* ATCC 22019 THL (2.269)
	Kodamaea (Pichia) ohmeri	*Pichia ohmeri* MY970_1_09 ERL (1.999)
	Candida orthopsilosis	*C. orthopsilosis* P3119_8_37 HAC (2.059)
	Saccharomycopsis fibuligera	No reliable identification

P. pentosaceus, *L. brevis*, *W. cibaria*, *W. paramesenteroides*, *Leu. citreum*, *Leu. mesenteroides*, and *Lc. lactis*, and the fungi identified were *Sc. fibuligera*, *H. burtonii*, *K. ohmeri*, *R. oryzae/A. rouxii*, *M. indicus*, and *C. intermedia*. The culture-dependent methods detected four LAB, namely *P. pentosaceus*, *L. brevis*, *L. fermentum*, and *L. plantarum*, as well as the fungi *Sc. fibuligera*, *H. burtonii*, *K. ohmeri*, *P. anomala*, *C. parapsilosis*, and *C. orthopsilosis*.

Overall, the culture-independent method identified seven LAB and six fungal species, whereas the culture-dependent methods identified only four LAB and seven fungal species. Both types of method were able to detect the presence of *P. pentosaceus*, *L. brevis*, *Sc. fibuligera*, *H. burtonii*, and *K. ohmeri*. PCR-DGGE seemed to favor the detection of *W. cibaria*, *W. paramesenteroides*, *Leu. citreum*, *Leu. mesenteroides*, *Lc. lactis*, *R. oryzae/A. rouxii*, *M. indicus*, and *C. intermedia*. In addition, SDS-PAGE and MALDI-TOF MS could identify culturable microorganisms such as *L. fermentum*, *L. plantarum*, *P. anomala*, *C. parapsilosis*, and *C. orthopsilosis*.

Our data indicate that each method favors the identification of certain species. In this regard, the culture-independent method is most suitable for monitoring the general distribution of LAB and fungal communities. This method could be used to predict whether multiple samples originated from the same region or have similar functions, although minor inconsistencies in the detected species will invariably be present. On the other hand, the culture-dependent methods are not as suitable for determining tapai origin. This is due to the nature of uncontrolled tapai preparation, which results in inconsistencies in the presence of detected microorganisms and in the number of isolates, even when the samples are manufactured from the same company or originate from the region. In addition, our data clearly indicate that laru preparation does not always take place under well-controlled and standardized conditions, as demonstrated by the findings from region B (samples 3, 5, 6, 7, 9, 13, 14, 16, and 17), region K (samples 2, 6, and 11), and region T (samples 12 and 15). However, the combination of both approaches can provide an overall view of the laru microbiota.

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